Comparison of PTH and Hypocalcin in Mammalian and Teleost Bioassays

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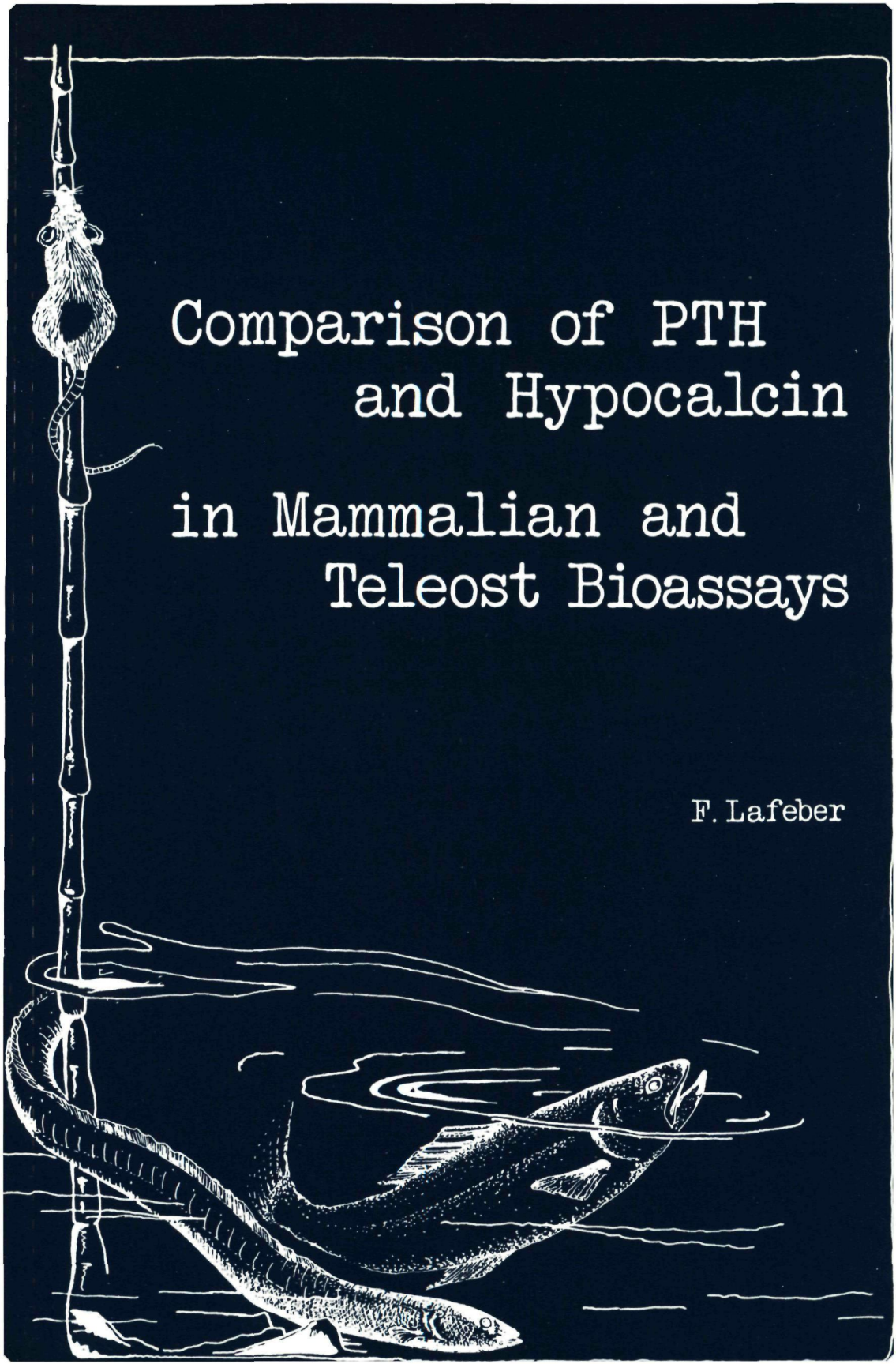

COMPARISON OF PTH AND HYPOCALCIN IN MAMMALIAN AND TELEOST BIOASSAYS

COMPARISON OF PTH AND HYPOCALCIN IN MAMMALIAN AND TELEOST BIOASSAYS

een wetenschappelijke proeve op het gebied van
wiskunde en natuurwetenschappen

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Opgedragen aan Marja en Rik

CONTENTS

GENERAL INTRODUCTION

This study deals with the isolation, structure and function of hypocalcin, a hormone present in fish. Hypocalcin has been suggested to be homologous to parathyroid hormone (PTH), which was mainly based on immunoreactivity. Hypocalcin is the principle factor produced in the Stannius bodies (corpuscles of Stannius, CS), small endocrine glands present only in holostean and teleostean fish. It is considered the major hypocalcemic hormone in these animals. Parathyroid glands are present only in terrestrial vertebrates and PTH is the principle hypercalcemic hormone. The suggested homology of hypocalcin and PTH, therefore, would imply that the hypocalcemic hormone of fish has been turned into a hypercalcemic hormone during the water to land transition in vertebrate evolution. Such a functional change is only understandable when the regulation of calcium metabolism differs substantially between aquatic and terrestrial vertebrates, and this indeed has been established (1).

The main calcium regulating organs in terrestrial vertebrates are gut, kidney and bone. The gut is involved in the uptake of calcium, whereas the kidneys are involved the secretion and reabsorption of calcium. Bone is important as a calcium buffer in terrestrial vertebrates, since calcium uptake *via* the food is intermittent. The excess of ingested calcium has to be stored in the bone, whereas a shortage of calcium uptake can be replenished with calcium resorbed from the bone. The deposition and resorption of calcium in bone is mediated by osteoblasts, osteocytes and osteoclasts.

In terrestrial vertebrates endocrine calcium regulation is mainly under control of parathyroid hormone (PTH), vitamin-D_3-metabolites (Vit-D_3) and calcitonin (CT). Vit-D_3 and PTH are involved in hypercalcemic regulation, including an increased calcium uptake by the gut, reabsorption *via* the kidney and resorption from the bone. CT on the other hand is, by effecting these targets, involved in hypocalcemic control. The interplay of these mechanisms allows very precise regulation of blood calcium concentrations in the terrestrial vertebrates. (2)

Fish are also able to maintain body fluid calcium concentrations within narrow limits, and precise regulation of plasma calcium has been observed in all species investigated (3, 4). In contrast to terrestrial vertebrates, fish take up calcium directly from the water *via* the gills, and only for a minor part *via* the gut (5). Regulation of the uptake of calcium seems, therefore, to occur mainly at the gill level (6). Although both gut and kidney are considered as possible targets for calciotropic hormones in fish, the literature is scarce and shows many contradictions (7-9). Whereas in terrestrial vertebrates the importance of bone and bone cells for calcium regulation is undisputed, it is still unknown to what extent bone participates in fish plasma calcium regulation. There is great diversity in the degree of ossification of fish bone. Some species have hardly any mineralized tissue, whereas others have an extensively mineralized skeleton (4, 10, 11). Moreover, some species have cellular bone (with enclosed osteocytes) whereas others have acellular bone. Bone resorbing cells have been observed in only a few species (1). Furthermore, fish can take up calcium continuously from the water and this seems to diminish the necessity of a rapidly accessible internal calcium buffer. It is therefore unlikely that the role of bone in fish calcium regulation is as important as it is in terrestrial vertebrates.

Because fish live in an aquatic environment with calcium concentrations several times lower (freshwater) to several times higher (seawater) than that of the body fluids (12) and calcium uptake mainly takes place *via* the gills, fish need appropriate calcium regulating mechanisms to maintain blood calcium concentrations within physiological limits. It is therefore not surprising that endocrine factors involved in calcium regulation in fish are different from those of the terrestrial vertebrates.

Parathyroid glands have never been observed in fish (1). In the absence of PTH hypercalcemic control in fish may be achieved by prolactin secreted from the pituitary gland (12, 13), although in some species hypercalcemic effects of cortisol have been reported (14). However, whereas PTH is a fast acting hypercalcemic hormone in terrestrial vertebrates, in fish it takes days to obtain a calcemic response after prolactin administration (15). There is another

hypercalcemic factor in the fish pituitary, which shows acute effects on fish calcium levels (15-17). Unfortunately, the identity of this hypercalcemic principle is still unknown. Several Vit-D_3-metabolites, including the important 1,25$(OH)_2$-D_3 have been demonstrated in fish (18). The function of these metabolites in calcemic control in fish however, is still in discussion. Calcitonin is probably of minor importance in hypocalcemic regulation of plasma calcium levels in fish, although salmon calcitonin is known for its clinical applications (19). Hypocalcemic control in fish is reported to be achieved by the CS (20). Removal of the CS results in extreme hypercalcemia which can be reduced by injections of homogenates of the glands (20). On the basis of this effect, the hormonal substance present in CS homogenates of some teleost fish has been called hypocalcin by Pang and coworkers (21). Ma and Copp have isolated a peptide fragment from salmon CS that they called teleocalcin (22). The biological activity of this factor, however, has not been established beyond doubt. It may be evident that, compared to the terrestrial vertebrates, endocrine calcium regulation in fish is less well defined. In particular the identity and function of the CS hormone is unclear.

The suggested similarity between hypocalcin and PTH and the scarcity of information on identity and function of hypocalcin prompted us to test the relationship between the corpuscles of Stannius and the parathyroid glands. Furthermore, we were interested in the mechanisms involved in the hypocalcemic control of fish. For this reason the effects of hypocalcin and PTH were compared in mammalian and fish bioassays (Part I). Additionally, the physiological function of hypocalcin was studied by analyzing its effects on gill calcium uptake (Part II).

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THE RELATIONSHIP BETWEEN THE CORPUSCLES OF STANNIUS AND THE PARATHYROID GLANDS

(Part I)

INTRODUCTION

The question whether there is a relationship between the CS and PTH was raised because of the following cosiderations.

Although parathyroids have never been found in fish, there has been interest in the activity of mammalian parathyroid extracts (PTE) in fish since 1958 (1). Bioactivity of PTH in fish assays might implicate the presence of a PTH analogue in fish. Several experiments have been reported that show effects of PTH and parathyroid extracts (PTE) in fish. After PTH injections in fish, increases as well as decreases of plasma calcium levels were shown, and effects on bone deposition and as well as on bone resorption were reported (1-7). More recently, this work has been resumed. Leung and Fenwick (8) showed a hypocalcemic effect of CS extracts in rat. In contrast, Milet and colleagues (9) reported a hypercalcemic effect of CS extracts in rat. Effects of CS extracts in anurans and in birds were shown to be biphasic; within the first 4 hours after administration of CS extracts a hypocalcemic effect was observed, which was subsequently followed by a hypercalcemic response (10, 11). Thus, effects have been reported of PTH in fish and of CS extracts in terrestrial vertebrates, although there is no consistency in the results reported.

Lopez, Milet and colleagues found some indications for a structural similarity between PTH and the principle from the CS. They tested PTH-antisera on blood plasma of intact eels and in eels after the removal of the CS (stanniectomy; STX), and on CS of control and $CaCl_2$ injected eels. Cross-reactivity was found and the CS identified as the source of this PTH-like principle (12, 13). However, cross-reactivity of PTH antisera with head-kidney and pituitary tissue was also reported (see 14). Notwithstanding, the immunological similarity between the CS principle and PTH was interesting and formed part of the basis for the work described in this section of my thesis.

The mode of control of hormone release from the CS also shows some similarity with that of the parathyroids, although the mechanisms involved have not been established with certainty. The glands are well

vascularized and innervated. The endocrine activity of the CS *in vitro* is increased (directly or indirectly) by elevated blood calcium concentrations (15). Aida (16) showed that *in vitro* the CS respond directly to changes in extracellular Ca^{2+} concentrations. Nervous control of hormone release is probably indirectly effected by adrenergic control of the vascular flow through the CS (14). Parathyroid glands are also well vascularized and innervated, although it has been suggested that they are less well innervated than the CS (14). Hormone release from the parathyroids is for the larger part under direct control of the extracellular fluid Ca^{2+} concentration (17). In similarity with the CS, nervous control of hormone release seems to be established by adrenergic control of the parathyroid gland blood flow (18, 19).

Both the CS and the parathyroids are involved in the control of blood calcium levels. Whereas the parathyroids are involved in hypercalcemic control, the CS are involved in hypocalcemic control. Removal of the parathyroids from terrestrial vertebrates results in hypocalcemia which can be reversed by injections of parathyroid tissue extracts (20). On the other hand, STX has been reported by many authors and for several fish species to result in hypercalcemia, whereas re-implantations or injections of crude tissue extracts reverse the STX induced hypercalcemia (*e.g.* 21, 22). Some authors have reported that STX also induces changes in blood electrolytes other than calcium. These effects, however, may be indirect and result from the rise in the plasma calcium concentration after STX (23, 24).

Similarity between CS and the parathyroid glands is further indicated by the presence of immunoreactivity in the CS of eels to antibodies raised against secretory protein I (SP-I; 25). SP-I and related peptides are cosecreted in several types of polypeptide producing endocrine cells that are derived from the neural crest, like the parathyroids. Although embryological similarity has been questioned (14), the presence of an SP-I like protein in the CS suggests that both glands have a similar embryological origin.

These reported similarities between the CS and the parathyroid glands prompted us to further investigate the relationship between their respective hormones, hypocalcin and PTH.

In this part of my thesis it is shown that a CS tissue extract contains a principle able to induce bone resorption in a way similar to PTH (Ch. 2). This principle (hypocalcin) was isolated and identified (Ch. 3) and subsequently shown to be responsible for the PTH-like bone resorbing activity of the CS extracts (Ch. 4). Furthermore, hypocalcin and PTH were shown to decrease plasma calcium levels in CS deprived eels (Ch. 5). Although we observed striking similarities in bioactivity between hypocalcin and PTH, the observed difference in amino acid sequence between both hormones makes a homology between the CS and the parathyroids unlikely.

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PTH-LIKE EFFECTS OF RAINBOW TROUT STANNIUS PRODUCTS ON BONE RESORPTION OF EMBRYONIC MOUSE CALVARIA *IN VITRO*.

ABSTRACT

Products of the Stannius corpuscles (CS) of rainbow trout were tested in an established parathyroid hormone (PTH) bioassay involving bone resorption in embryonic mouse calvaria. Aqueous extracts from Stannius corpuscles (CS-homogenate) showed a bone resorbing activity comparable to PTH in 24 h cultures of calvaria, indicated by a dose-dependent stimulation of lactate production and of calcium, phosphate as well as β-glucuronidase release. Moreover, CS-homogenates induced an increase in osteoclastic activity. The PTH-like CS-principle is released during *in vitro* incubations of the glands. These results and the lack of an additive effect of CS products and PTH on bone resorption suggest that both products activate the same receptor. We hypothesize that the hypocalcemic hormone of the CS of fish shares structural resemblance with PTH, the major hypercalcemic hormone of terrestrial vertebrates.

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Stannius corpuscles (CS) are small endocrine glands characteristic of holostean and teleostean fish. Rainbow trout usually have 2 to 5 ovoid corpuscles -located ventrocaudally to the kidney- that vary in diameter from 2 to 3 mm (1). For several species it has been reported that two different endocrine cell types are present (1-5). There is ample evidence that the predominant cell type produces a hypocalcemic factor, whereas the second cell type may be involved in sodium and potassium regulation (6,7). Effects of CS-products on plasma phosphate, magnesium and chloride have been reported, but these effects are usually rather small and may result from a disturbance of calcium metabolism (6,7). Plasma calcium concentrations rise considerably after stanniectomy (STX); CS implants or injections of CS-extracts completely reverse STX induced hypercalcemia (8,9).

Structural resemblance has been suggested between the hypocalcemic factor of the CS and parathyroid hormone (PTH), a hormone considered typical for terrestrial vertebrates and absent in fish. Antibodies raised against bovine PTH(1-84) showed cross-reactivity with a substance in eel blood plasma. This substance disappeared from the blood after STX and increased after the infusion of calcium chloride (10). Also, secretory substances of the CS cells have been shown to cross-react with antibodies raised against bPTH. Overloading the blood with calcium chloride resulted in the release of anti-bPTH positive secretory material (11).

The immunological similarities between the CS principle and PTH prompted us to investigate a possible relationship in bioactivity between PTH and the CS principle.

In higher vertebrates PTH is known to stimulate bone resorption. For rodents the effects of pharmacological doses of PTH on several parameters for bone resorption *in vitro* have been thoroughly investigated. Typically, PTH induced bone resorption is accompanied by an increased lactate production, as a result of enhanced aerobic glycolysis (12). PTH induced bone resorption results from an increase in the number of osteoclasts as well as in their bone resorbing activity (13). Increased osteoclastic activity and increased release of

lysosomal enzymes (*e.g.* β-glucuronidase; 14) as well as the removal of bone mineral components (mainly calcium and phosphate; 12) may serve, therefore, as parameters for PTH-induced bone resorption.

We used embryonic mouse calvaria -a tissue with considerable PTH-sensitive bone resorbing activity (12)- to characterize the bioactivity of the CS principle, using the forementioned parameters.

MATERIALS AND METHODS

Calvarium culture technique.

Calvaria were removed from 18 day old mouse embryos and each calvarium was bisected. The left half of one calvarium and the right half of the second calvarium and *vice versa* were fixed at 2 cm from the bottom in a 20 ml roller-tube, containing 1 ml of culture medium. The tubes were placed in an almost horizontal position in a roller-drum for 24 h (6-7 rotations per h) (15). The culture medium consisted of 90% Hanks'balanced salt solution (Hanks'BSS) and 10% heat inactivated human serum (h-serum). Total calcium and phosphate concentrations in this medium were 2.7 and 1.8 mM, respectively. After 24 hours of incubation at 37 °C, calvaria were removed from the incubation medium that was analyzed within 24 hours for calcium, phosphate, lactate and β-glucuronidase activity. Media were stored, frozen at minus 20 °C; the activity of β-glucuronidase has been reported not to decrease over a 24 h period (16).

Hormone administration

CS homogenate. Stannius corpuscles (a generous gift from Dr N. Mayer-Gostan; Laboratoire Jean Maetz; Villefranche-sur-Mer, France) were collected from rainbow trout (*Salmo gairdneri*); whole glands were lyophilized and stored at minus 60 °C. Approximately 25 mg lyophylized material (equivalent to about 100 mg wet weight CS, the amount of tissue obtained from 10 kg trout) was homogenized in 2.5 ml ice-cold Hanks BSS. After centrifugation (15 min; at 9.000*g*) the supernatant was diluted to desired concentrations with culture medium, and used within 2 h. Doses were expressed in mg wet weight of homogenized CS per ml calvarium culture medium.

Collection of CS products released in vitro. After a minimum acclimation period of 2 weeks to Nijmegen tapwater (0.8 mM Ca), rainbow trout were killed, and the CS removed, weighed and rinsed in Hanks'BSS. Subsequently they were incubated for 5 hours at 22 °C in Hanks'BSS in a shaking water-bath (approximately 10 mg wet weight/150 µl Hanks'BSS). The incubations were terminated by removal of the corpuscles. The media were quickly frozen in liquid N_2 and stored at -60 °C, for a maximum of 7 days. These media were thawed shortly before use and diluted to desired concentrations with culture medium. In these experiments only the calcium concentration of the medium was determined as a measure for bone resorption and an experimental set up was used that allowed paired observations as described elsewhere (15). CS doses were expressed as mg wet weight -used for incubation- per ml calvarium culture medium.

Parathyroid hormone. Bovine PTH was purchased from Sigma (TCA powder; Cat.no: p0892; 140 IU/mg) and dissolved in 0.005 N acetic acid containing 1% Pentex-albumin. Solutions of 1 IU/µl were stored in liquid N_2. For the parameters tested in this study no differences were observed between the effects of this purified PTH and synthetic b-PTH(1-34) (12). Immediately before use, PTH was diluted to desired concentrations with culture medium. Doses are expressed as IU PTH per ml of calvarium culture medium.

Controls. CS-homogenates were heat-treated by a 15 min incubation at 100 °C. Bone resorbing activity of such heat-treated homogenates was tested using forementioned biochemical parameters. In another set of experiments 100 mg tissue samples (wet weight) of rainbow trout liver, brain, muscle and trunk kidney were removed and homogenized in 1 ml ammonium acetate (50 mM, pH 7.4). After centrifugation (5 min, 9000*g*) supernatants were lyophylized and stored at -20 °C. For experiments the samples were dissolved in culture medium to a concentration of 5 mg wet weight CS per ml culture medium. Release of calcium and phosphate was determined as a measure for bone resorption.

Analytical methods.

Medium total calcium content was determined with a commercial colorimetric calcium-kit (Sigma, Cat.no: 586). Inorganic phosphate was measured according to the method of Delsal and Manhourim (17). Combined calcium phosphate standards (Sigma Cat.no: 360-11) were used as

reference. The medium lactate concentration was measured as described by Lowry et al.(18), using an auto-analyzer method (19). Lithium lactate (Sigma) was used as reference. The activity of β-glucuronidase in the culture medium was determined according to Mead et al. (20), using the conversion of 4-methyl-umbelliferyl-glucuronide (Boehringer-Mannheim) to 4-methyl-umbelliferyl during a 30 min incubation period at 37 °C; 4-methyl-umbelliferyl (BDH) was used as a reference.

Histology.

For light microscope examinations, calvaria were incubated as described above. After a 24 h incubation period, calvaria were fixed in phosphate buffered (pH 7.0) 4% glutaraldehyde solution (24 h; 4 °C) and decalcified in 5% formic acid, containing 5% formaldehyde (3 h at 4 °C), followed by two washes in distilled water. Subsequently, acid-phosphatase staining was performed according to Barka and Anderson (21). Calvaria were embedded in paraffin; 5 µm-sections were stained with acid haematoxylin. Serial sections from calvarium halves were used to quantify osteoclast nuclei; in every fourth section nuclei surrounded by acid-phosphatase positive cytoplasm were scored. Results are expressed as the mean number of osteoclast nuclei per section ± SEM for 3 calvarium halves.

Statistical analysis.

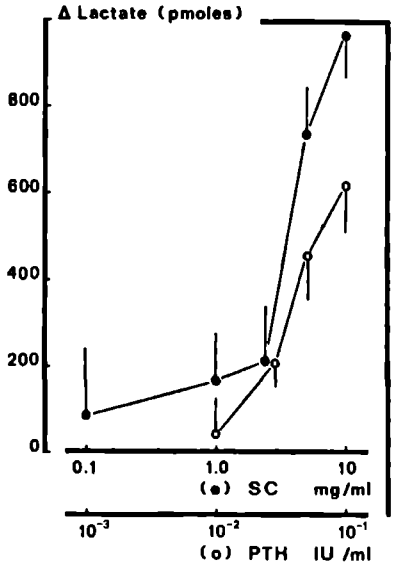
Stastistical evaluation was performed by the use of the Mann-Whitney *U*-test (one-tailed). For paired observations Student's *t* test was used. Significance was accepted at $P < 0.05$. Mean values ± SEM are given.

RESULTS

Biochemical observations.

Lactate production. Figure 1 shows the relationship between the increase in lactate production by calvaria and the concentrations of CS-homogenate or PTH. CS-homogenate and PTH yielded a similar activation pattern. Proceeding from an equipotent bioactivity of 10 mg

of CS tissue and 0.1 IU PTH, no significant difference between the degree of stimulation by CS-homogenate or by PTH could be observed. At the concentrations CS-homogenate tested no maximum lactate production was observed. This observation corroborates data that we obtained for



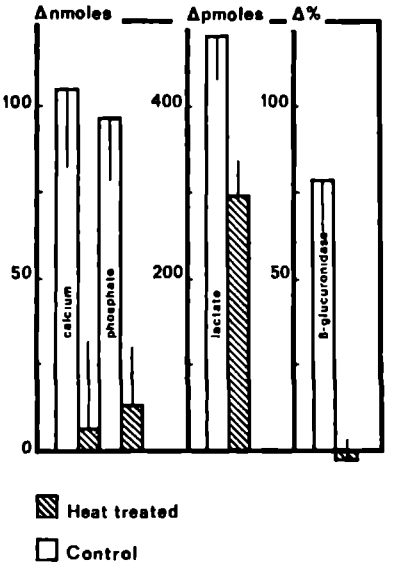


Figure 1. Effects of CS-homogenate (●---●) and PTH (0---0) on lactate production by 2 calvarium halves, cultured for 24 h. Mean values ± SEM are given; for CS-homogenate n=16-24 and for PTH n=4-16. Significant stimulation of lactate production occurred at concentrations above 2.5 mg/ml CS-homogenate or 10^{-2} IU/ml PTH.

Figure 2. Lactate production, calcium, phosphate and β-glucuronidase release induced by untreated and heat inactivated CS-homogenate (10 mg/ml), during a 24 h incubation period. Mean values ± SEM are given; n = 8; all differences with the controls are statistically significance.

the effects of PTH on embryonic mouse calvaria. As much as 1 IU PTH per ml is needed to obtain a maximum lactate production (12). Proceeding from a similar pattern for PTH and for CS-homogenate induced lactate production, it may be calculated that at least 100 mg CS-homogenate per ml medium would be required to produce maximum lactate production.

Heat treatment of CS homogenates significantly deminished lactate production, although the stimulatory effect on lactate production by CS homogenates was not completely abolished (Fig. 2).

Bone demineralization. Figure 3 shows a dose-response curve for CS-homogenate and PTH induced calcium release from embryonic mouse calvaria. An increase in the amount of CS product resulted in an increase of calcium resorption from the bone. As shown in Figure 4, a similar dose-response relationship was found for CS-homogenate and phosphate release from calvaria. Using calcium and phosphate release as

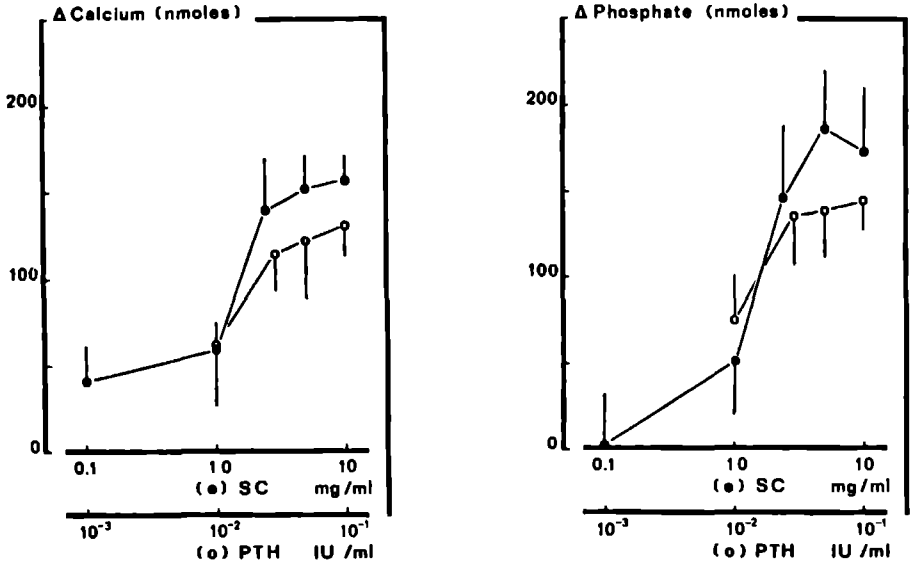


Figure 3. Effects of CS-homogenate (●---●) and PTH (0---0) on calcium release from 2 calvarium halves, cultured for 24 h. Mean values ± SEM are given; for CS-homogenate n=16-24 and for PTH n=4-10. Significant stimulation of calcium release occurred at concentrations above 1.0 mg/ml CS-homogenate and all PTH concentrations tested.

Figure 4. Effects of CS-homogenate (●---●) and PTH (0---0) on phosphate release from 2 calvarium halves, cultured for 24 h. Mean values ± SEM are given; for CS-homogenate n=16-24 and for PTH n=4-16. Significant stimulation of phosphate release occurred at concentrations above 1.0 mg/ml CS-homogenate and all PTH concentrations tested.

parameters for bone resorption, again 10 mg CS wet weight could be equated with about 0.1 IU PTH. The maximum amounts of calcium and phosphate, released by CS homogenate after a 24 h cultivation period, were similar to the values observed for the maximum release induced by PTH. Half-maximum calcium and phosphate release occurred at about 2.5 mg CS wet weight/ml, and at about $2.5 \cdot 10^{-2}$ IU/ml PTH. The value for PTH is in good agreement with data from the literature (12).

Maximum stimulation of calcium and phosphate release was obtained by the addition of 0.1 IU PTH or 10 mg wet weight of CS-homogenate separately. No further increase in calcium and phosphate release could be observed when maximally stimulating doses of CS-homogenates and PTH were added together (Table 1).

Table 1. Calcium and phosphate release induced by maximum effect concentrations of CS-homogenate and PTH added separately or together.

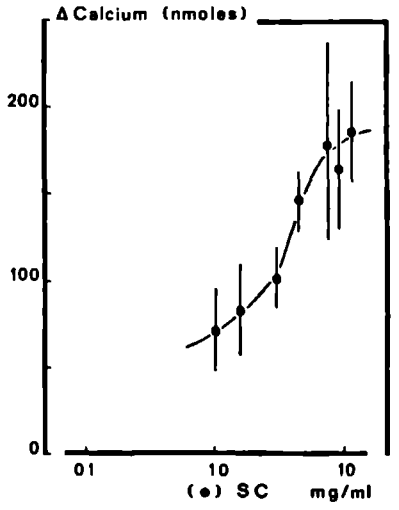
	Δcalcium release (nmoles)	Δphosphate release (nmoles)
Stannius (10 mg/ml)	125 ± 44 *(6)*	119 ± 28 *(9)*
PTH (10^{-1} IU/ml)	154 ± 33 *(9)*	91 ± 28 *(9)*
Stannius + PTH	147 ± 30 *(10)*	117 ± 16 *(10)*

Mean values ± SEM are given with the number of observations in parentheses. No statistical significant differences were observed.

Heat treatment of CS-homogenates resulted in a complete loss of the ability to demineralize the calvaria (Fig. 2).

Bone resorption in mouse calvaria was also induced by the products that are released during *in vitro* incubation of CS. In Figure 5 the dose-response relationship for CS secretory products and calcium release is shown. A similar potency to stimulate calcium release was found for CS-homogenates and the products released by the same amount of tissue during a 5 h incubation period.

β-glucuronidase release. Figure 6 shows the dose response relationship for β-glucuronidase release induced by CS-homogenate and



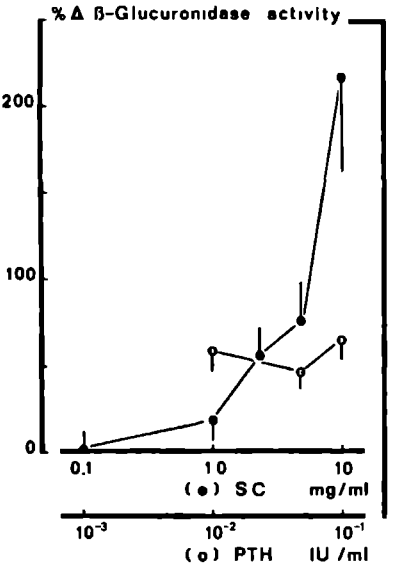


Figure 5. Calcium release from 2 calvarium halves, cultured for 24 h, induced by CS-products secreted during *in vitro* incubations. Mean values ± SEM are given; paired observations (n=3-6). Stimulation at concentrations above 1 mg/ml CS (wet weight used for *in vitro* incubation) are statistically different from the release in the absence of hormone.

Figure 6. CS-homogenate and PTH induced stimulation of the β-glucuronidase activity, during a 24 h culture period. Values ± SEM are expressed in % ; release in the absence of hormone was equated with 100 %. For CS-homogenate n=16-24 and for PTH n=4-14. The increase in the release of β-glucuronidase activity at concentrations above 1.0 mg/ml CS-homogenate, and at all PTH concentrations tested are statistically different from activity in the absence of hormone.

PTH. An increasing amount of CS-homogenate resulted in an increasing release of β-glucuronidase. The release of β-glucuronidase was stimulated by PTH over a range of 0.01 to 1 IU/ml. At a concentration of 0.3 IU/ml and 1 IU/ml we found 83 ± 22 % and 62 ± 10 %, respectively (data not shown in Fig. 5). No dose dependency was observed for PTH

induced β-glucuronidase release at the concentrations tested. The activation pattern of PTH differed from the CS-homogenate activation pattern.

Heat treatment of the CS-homogenates resulted in a complete loss of the ability to increase the β-glucuronidase release of calvaria (Fig 2.).

Table 2. Calcium and phosphate release induced by tissue homogenates of trout CS, kidney, muscle, brain and liver (5 mg per ml).

5 mg/ml	Δ Calcium (nmoles)	Δ Phosphate (nmoles)
Stannius	153 ± 19 *(23)* *	186 ± 37 *(24)* *
Kidney	-22 ± 48 *(14)*	37 ± 27 *(13)*
Muscle	-29 ± 25 *(11)*	8 ± 17 *(13)*
Brain	-62 ± 14 *(9)*	-12 ± 15 *(13)*
Liver	-6 ± 15 *(13)*	-28 ± 34 *(13)*

Mean values ± SEM are given with the number of observations in parentheses. Asterisks indicate statistical significance.

Control homogenates. Neither calcium- nor phosphate-release was significantly stimulated by tissue homogenates of trout liver, brain, muscle and kidney (Table 2).

Histological observations.

Osteoclast activation. The addition of CS-homogenate as well as of PTH to the incubation medium of the calvaria resulted in a significant increase in the number of osteoclast nuclei after a 24 h incubation period (Table 3). Doses of PTH and CS-homogenate, giving maximum calcium and phosphate release, were tested. At these doses, no significant difference was observed between the effect of CS-homogenate or PTH.

Table 3. Effects of CS-homogenate and PTH on the calvarium osteoclast activity.

	number of nuclei per section (in %)			
Control	100	±	10	*(6)*
CS-homogenate (5mg/ml)	158	±	22*	*(3)*
CS-homogenate (10mg/ml)	178	±	31*	*(3)*
PTH (1 IU/ml)	183	±	1 *	*(2)*

The number of nuclei per section of calvarium halves incubated in the absence of hormones is equated with 100 %. Mean values ± SEM are given with the number of observations in parentheses. Asterisks indicate statistical significance.

DICSUSSION

We conclude from the present data that the corpuscles of Stannius of rainbow trout produce and secrete a product that resembles PTH in its bone resorbing action. Aqueous extracts from CS as well as products released by these glands *in vitro* stimulate bone resorption in embryonic mouse calvaria in a way comparable to PTH. Lactate production as well as calcium and phosphate release were stimulated dose-dependently. The patterns of lactate production and the release of calcium and phosphate were essentially similar for CS-homogenate and PTH stimulated samples. In this bioassay 100 mg wet weight CS (the amount of tissue obtained from 10 kg trout) was found to be equivalent to 1 IU PTH. In our assay on the release of β-glucuronidase, however, CS-homogenate proved to be more stimulatory than PTH. The relatively weak and dose-independent stimulatory activity of PTH over this concentration range is not clearly understood. For a comparable bioassay as used in this study, Vaes (14) reported a linear correlation between mineral release and β-glucuronidase release, during 24 to 72 h incubations with 0.01 to 1 IU/ml PTH. We found a comparable relationship between β-glucuronidase and phosphate release after stimulation with CS-homogenate. For PTH, however, such a relationship was not found. Quantitatively, our observations on PTH-induced

β-glucuronidase release (62 ± 10 % increase at 1 IU PTH) compare well with those of Vaes, who showed an increase of 75 % after the addition of 1 IU PTH (14). The specificity of the bone resorbing effect for the CS homogenates is indicated by the absence of an effect of kidney, liver, brain and muscle homogenates in this bioassay.

Most likely the PTH-like substance of the Stannius corpuscles is heat labile. Incubation of CS-homogenate at 100 °C resulted in a loss of its ability to stimulate the release of calcium, phosphate and β-glucuronidase. The stimulatory effect of CS products on lactate production, although significantly diminished by heat treatment, was not reduced to controle levels. Apparently, CS-homogenates contain a heat labile factor, involved in bone resorption as well as a heat stable factor, capable to induce lactate production but independant from bone resorption. For this bioassay it has been shown before that the lactate production can be stimulated without inducing bone resorption (12).

CS-products increase the number of osteoclast nuclei in mouse calvaria. After an incubation period of 24 h, we observed a similar increase in the number of osteoclast nuclei in the PTH and the CS-homogenate stimulated calvaria, at concentrations that induce maximum calcium and phosphate release. The method used in our experiments did not allow the determination of the number of osteoclasts or number of nuclei per cell separately. Whether the increase in the number of osteoclast nuclei is the result of an increase in the number of cells or in the number of nuclei per cell remains to be established. Rowe and Hausmann (22), however, have shown that both an increase in the number of nuclei per cell and an increase in the number of osteoclasts (which also results in an increased number of nuclei, but may result from a different stimulatory mechanism) may result in stimulated bone resorption. Our data with respect to PTH corroborate those of Holtrop and coworkers (23) who showed an increase in the number of osteoclasts in rat bones after a 24 h stimulation with PTH. With respect to CS-homogenates, our results corroborate histological observations by Milet *et al.* (24); they reported a stimulation of osteoclastic activity 0.5 h after injections of CS extracts in rat.

The CS principle responsible for bone resorption is released

during *in vitro* incubations of Stannius corpuscles. Recent studies in our laboratory give growing evidence for a proteinaceous product, with an apparent molecular mass of approximately 25 kDa, as the putative hormone of these glands (25).

Both PTH and the CS-principle exert their bone resorbing action via the same pathway; no additive effect was observed when maximum stimulating concentrations of PTH and CS homogenate were tested together. From the close resemblance between PTH and CS-products in the bioassay reported here, we suggest that the Stannius principle stimulates bone resorption via activation of the PTH receptor.

One could argue that the bone resorbing effects of CS homogenates reported in this paper originate from an action by prostaglandins. However the amount of prostaglandins extracted during our homogenization procedure (an aqueous extract) will be very small. More importantly, there is no reason to assume that CS contain more prostaglandins than any of the other control tissues tested.

Fontaine and coworkers (26,27) were the first to report -and it is generally accepted now- that the CS produce a factor that is hypocalcemic in fish. More recently, Lopez and coworkers (10,11) suggested, that this hypocalcemic factor was homologous with PTH. This suggestion was mainly based on immunocrossreactivity of the presumed CS hormone with PTH antisera (10,11). This immunological resemblance should be considered with caution, however. It has been shown that antisera against PTH crossreact with products present in other endocrine tissues in fish (28). Moreover, not all PTH antisera tested cross-react with CS products (29), which indicates that the structural similarities between PTH and the CS product is only partial. On the other hand, in addition to the immunological resemblances and the remarkable similarity in *in vitro* bioactivity reported here, PTH and the putative CS hormone also show similar effects on blood calcium levels *in vivo*. Injections of crude CS-extracts and PTH both elevate blood calcium levels in rats (30), and reduce blood calcium levels in fish (25,31). All these data strongly suggest that both calcium regulating hormones have important structural similarities. An attractive hypothesis would be that these structural similarities result from phylogenetically conservative aminoacid sequences in the biologically active parts of PTH and the CS hypocalcemic hormone.

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IDENTIFICATION OF HYPOCALCIN (TELEOCALCIN) ISOLATED FROM TROUT STANNIUS CORPUSCLES

ABSTRACT

We have isolated and purified a glycoprotein from the corpuscles of Stannius (CS) of trout, which we consider hypocalcin, also called teleocalcin, the major hypocalcemic hormone of fish. This product is present in relatively large amounts in the CS of several species (*i.e.* European eel, tilapia, goldfish and carp). Hypocalcin is typically released from the CS in response to an experimentally induced increase of the blood calcium concentration. Ultrastructural observations show that after this treatment the type-1 cells, reportedly the hypocalcin producing cell type of the CS, are almost completely degranulated. The isolated glycoprotein has an apparent molecular weight of 54 kDa as determined by SDS-PAGE. This molecule appears susceptible to breakdown and is recovered upon concanavalin-A affinity chromatography as a 41 kDa product. Reducing agents such as mercapto-ethanol or dithiotreitol (DTT) employed, *e.g.* during standard electrophoretic techniques or during aminoacid sequence analysis, allow only the recovery of 28 kDa or 18 kDa products. Evidence is given that the 54 kDa and 41 kDa products are dimer molecules, with the 28 kDa and 18 kDa products as their respective monomeric constituents. The sequence of the first 33 N-terminal amino acids of these products and the composition of the sugar component are presented.

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Corpuscles of Stannius (CS) are endocrine glands characteristic for holostean and teleostean fish. Since their discovery by Stannius (1) a large body of histological research on the CS of many fish species has been carried out (2-5). CS produce a factor that that probably is the predominant hypocalcemic hormone in fish. Removal of the glands results in a strong increase in blood calcium concentration (*e.g.* 6) that is reversed by re-implantations of CS or injections of CS tissue homogenates (7-10). Since the gills probably form the major site of calcium exchange between fish and water, inhibition of branchial calcium uptake may underlie hypocalcemic control (11-13).

There exists immunological resemblance between the hypocalcemic principle of CS and the parathyroid hormone (PTH) of the higher vertebrates (14-16). Moreover, the CS hypocalcemic principle has similar bioactivity as PTH in mammalian and fish bioassays (Ch.2, 17-21). Although Wagner *et al.* (22) reported on a hypocalcemic principle isolated from salmon, much work remains on the characterisation and mechanism of action of the hypocalcemic principle in fish.

CS of freshwater and euryhaline species contain two different cell types, type-1 and type-2 cells, the latter being absent in most seawater species (see review by Wendelaar Bonga and Pang, 5). The fact that type-1 cells respond to changes in external calcium has led many researchers to postulate that these cells produce the hypocalcemic factor (23, 24, 5).

In 1976 Krishnamurthy (3) suggested, on the basis of histological staining by periodic acid Schiff reagent, that the type-1 cells contain a glycoprotein. In 1978 Ma and Copp (25) isolated a 3 kDa glycopeptide from salmon CS which they called teleocalcin; it was reported to be bioactive *in vitro*, by inhibition of branchial Ca^{2+}stimulated ATP hydrolyzing activity, and *in vivo*, by its hypocalcemic action in eel (26). Pang and coworkers (27), however, concluded from dialysis experiments that the bioactive factor of the CS of cod, which was called hypocalcin (10), was a product with a molecular weight of at least 13 kDa. Fenwick (28) provided evidence that the hypocalcemic principle of eels had a molecular weight of at least 10 kDa. In our

hands (29) the hypocalcemic principle of tilapia appeared as a 28 kDa product when analysed with electrophoretic techniques. Recently, Wagner *et al.* (22) succeeded in purifying and characterising a 39 kDa glycoprotein from salmon CS which showed inhibition of ^{45}Ca uptake from the water in juvenile rainbow trout. The group of Milet and coworkers, however, postulated the presence of two secretory substances in the CS. A 34 kDa peptide, which is called parathyrin of the corpuscles of Stannius (PCS), was partly purified and showed hypocalcemic bioactivity in stanniectomized eels (30). A 70-80 kDa glycoprotein showing immunocrossreactivity with chromogranin-A antisera and SP-1 (a secretory glycoprotein from the parathyroid cells) antisera was suggested to be cosecreted with PCS (31, 32).

In this paper we report on the purification and partial identification of rainbow trout hypocalcin. It will be shown that trout hypocalcin is a 54 kDa glycoprotein, that is readily released upon increased plasma calcium levels.

MATERIALS AND METHODS

Stannius tissue homogenates.

CS were obtained from rainbow trout (*Salmo gairdneri*), brown trout (*Salmo trutta*), European eel (*Anguilla anguilla*), tilapia (*Oreochromis mossambicus*), goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*). The glands were homogenized in 0.05 M ammonium acetate (pH 7.4) using a Potter homogenizer fitted with a teflon pestle. The supernatant obtained after centrifugation (5 min; 9000 x *g*) was lyophilized and prepared for SDS polyacrylamide gel electrophoresis (see below).

Calcium injection.

Rainbow trout and European eel were injected intraperitoneally with 0.68 M $CaCl_2$ solution (100 µl per 100 g fish per day) for two days. Injections of NaCl solutions of identical molality served as controls. Four hours after the last injection blood was collected by puncture of the blood vessels of the caudal peduncle using a heparinized syringe (Ca^{2+}-heparin, Radiometer). Subsequently the CS were removed and prepared for SDS-PAGE. Blood was analysed for ionic

calcium and pH; plasma was analysed for total calcium and osmolality. From every fish, part of the CS tissue was fixed and stained for electron microscopy as described by Wendelaar Bonga *et al.* (24). For the discrimination between type-1 and type-2 cells structural parameters as described by Wendelaar Bonga *et al.* (24) were used.

Analytical methods.

Plasma total calcium and phosphate was determined with a commercial calcium-kit (Sigma). Combined calcium/phosphate standards (Sigma) were used as a reference. Protein content was estimated with a commercial reagent kit (BioRad) using bovine serum albumin (BioRad) as a reference. Blood ionic calcium concentration and blood pH were determined using an automated ionic calcium analyser (ICA; Radiometer). Osmolality was measured using a Roebling micro-osmometer. Distilled water and an osmolality standard of 300 mOsmol/kg (Sigma) were used as standards.

SDS-polyacrylamide gel electrophoresis.

SDS-PAGE was performed according to Laemmli (33) with 15% polyacrylamide slab gels. To investigate the effects of reducing agents on the CS principle, SDS-PAGE was carried out under reducing conditions (with mercapto-ethanol) or non reducing conditions (without mercapto-ethanol). After fixation of the proteins in the gels by methanol and glutaraldehyde, the gels were silver-stained (34). Data were quantified by densitometric scanning using a BioRad model 1650 transmittance scanning densitometer.

Isolation procedure.

The isolation of the hypocalcemic principle from CS homogenates was started with concanavalin-A Sepharose-4B column chromatography (Sigma ; 1.77 cm2 x 5 cm). The column was equilibrated with 0.015 M Tris-buffer pH 7.4 containing $MnCl_2$, $MgCl_2$, $CaCl_2$, 1 mM each and NaCl, 1 M (con-A buffer) according to Roelfzema and Van Erp (35); the flow was 15 ml per hour at 4 °C. Lyophilized whole glands of rainbow trout were kept in stock at -20 °C. Approximately 100 mg dry weight tissue (equivalent to 400 mg wet weight obtained from 40 kg trout) was homogenized in 3 ml con-A buffer with a Potter-type homogenizer fitted with a teflon pestle. The supernatant obtained after centrifugation (5

min; 9000 x *g*) was applied to the column and passed three times through the column by short-circuiting the system. Products not bound to con-A (residue) were eluted with con-A buffer. The products binding to con-A were eluted with 0.3 M α-methyl-D-glucoside in con-A buffer (isolated product).

Desalting and concentration of eluted material were carried out by ultrafiltration (Amicon Inc, USA). Diaflo YM-10 membranes were used for the con-A binding material and YM-5 membranes for the residue. Ultrafiltration was carried out at 4 °C in 180 ml stirred cells operated at 2.6 MPa N_2. After the volume of the eluates had decreased to about 10 ml, the ultrafiltration cells were refilled with ammonium acetate buffer (0.05 M; pH 7.4) to 180 ml; this procedure was repeated three times. By so doing the con-A buffer was diluted over a thousand times. The remaining 10 ml was lyophilized in fractions as required for assays, and subsequently stored at -20 °C. SDS-PAGE of the samples was routinely carried out as a quality check for product composition. The samples containing the presumed hypocalcemic principle proved to be pure for at least 95% on protein basis.

HPLC-analysis of the con-A binding fraction was carried out using a ChromSpher C18 reversed phase HPLC column (Chrompack). Elution was performed with an acetonitrile gradient (0-50%) in a buffer containing sodium,potasium-tartrate (5 mM; pH 3.0), Na_2SO_4 (50 mM) and butanesulfonic acid (5 mM), the flow was 1 ml/min. Eluted material was detected with a Spectraflow-783 absorbance detector at 280 nm. The fraction containing a significant amount of protein was collected. Freeze drying and TCA precipitation (10 % final concentration) methods were used to remove acetonitril and salts from this samples before SDS-PAGE was performed.

Aminoacid sequence analysis.

Amino acid sequences were determined at the Gas Phase Sequenator Facility (Department of Medical Biochemistry, State University of Leiden, The Netherlands), The instrument used was an Applied Biosystems Model 470A Protein Sequencer, on-line equipped with a Model 120A PTH Analyser. Pyridyl-ethylation (36) was carried out for cysteine identification. Aminoacid sequence analysis was carried out in the presence of the reducing agent dithiotreitol (DTT).

Carbohydrate analysis.

Carbohydrate analysis of the isolated protein was performed at the University of Alberta, Department of Physiology, according to Dutton *et al.* (37). Data were quantified by gas liquid chromatography - mass spectrometry, using xylose as an internal standard.

Statistical analysis.

Data are presented as mean values ± SD. Statistical evaluation was performed by the use of the unpaired Student's *t*-test. Significance was accepted at $P < 0.05$.

RESULTS

Identification of the hypocalcemic factor.

Figs 1 and 2 show typical examples of densitometric scans of silverstained products, after SDS-PAGE under reducing conditions, present in a crude tissue homogenate of CS of six different teleost fish. A product with an apparent molecular weight of approximately 28 kDa was consistently observed. In Fig 2 it is shown for trout and eel that the 28 kDa protein disappeared almost completely from CS tissue homogenates after $CaCl_2$ injection. Typically, the 28 kDa protein constitutes 20% of the total amount of protein present in the CS tissue homogenate of these fish, as derived from analysis of the area under the curve of the densitometric scans of the silverstained SDS-gels. A densitometric scan after SDS-PAGE of a crude tissue homogenate of NaCl injected fish did not differ from a control CS tissue homogenate.

Electron micrographs (Fig 3 and Fig 4) show that the CS of the untreated and the NaCl injected fish contain an abundance of large secretory granules. These granules are typical for the type-1 cells (38, 23). The cells of the CS of the $CaCl_2$ injected fish are almost devoid of these secretory granules.

Total plasma calcium levels increased significantly in the $CaCl_2$ injected trout and eel (2.47 ± 1.12 to 3.10 ± 0.30 and 3.08 ± 0.16 to 6.14 ± 0.41 mM, respectively), but were unchanged in the NaCl injected controls. Plasma osmolality and blood pH did not change significantly in either group.

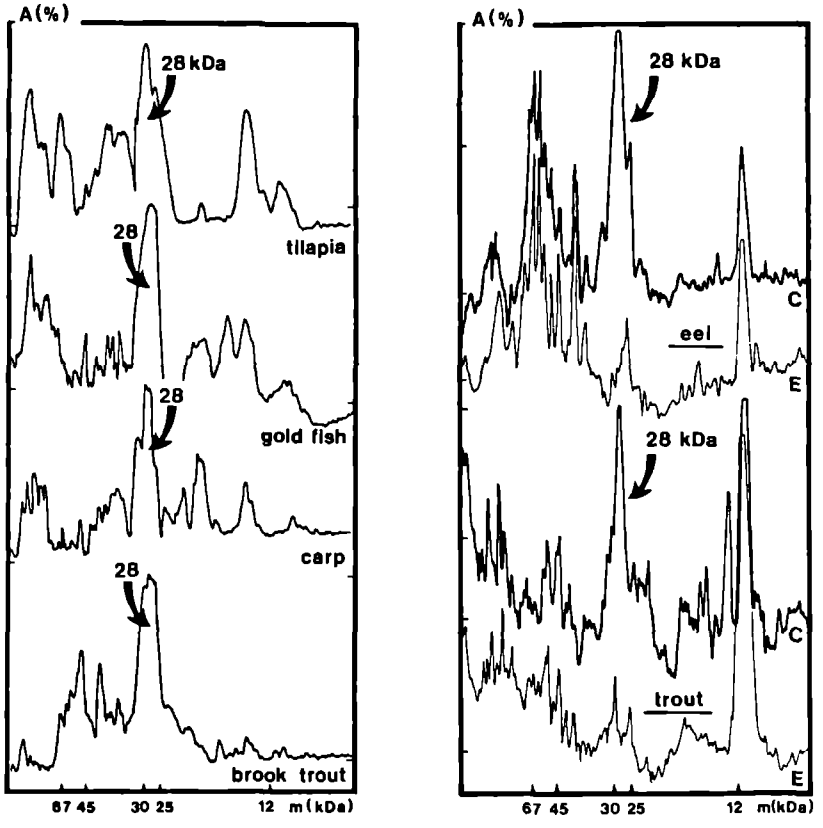

Fig 1. Densitometric scan of silverstained CS crude homogenates after SDS-PAGE of goldfish, carp, tilapia and brook trout. Arrow indicates 28 ± 2 kDa peak for all species. Absorbance is shown as percentage of maximum peak hight (=100%) and corresponds to the amount of material. Molecular weights (*m*) of protein markers are given on the horizontal axis.

Fig 2. Densitometric scan of silverstained CS crude homogenates of NaCl (C) or $CaCl_2$ (E) injected eel and trout after SDS-PAGE. Absorbance is shown as percentage of maximum peak height (=100%). Molecular weights (*m*) of protein markers are given on the horizontal axis.

Fig 5 shows a crude tissue homogenate of trout after SDS-PAGE under reducing and non-reducing conditions. Fig 5 (top) shows that this product has an apparent molecular weight of 28 kDa only after electrophoresis under reducing conditions. Surprisingly, under non-reducing conditions this product showed an apparent molecular weight of aproximately 54 kDa (Fig 5 bottom).

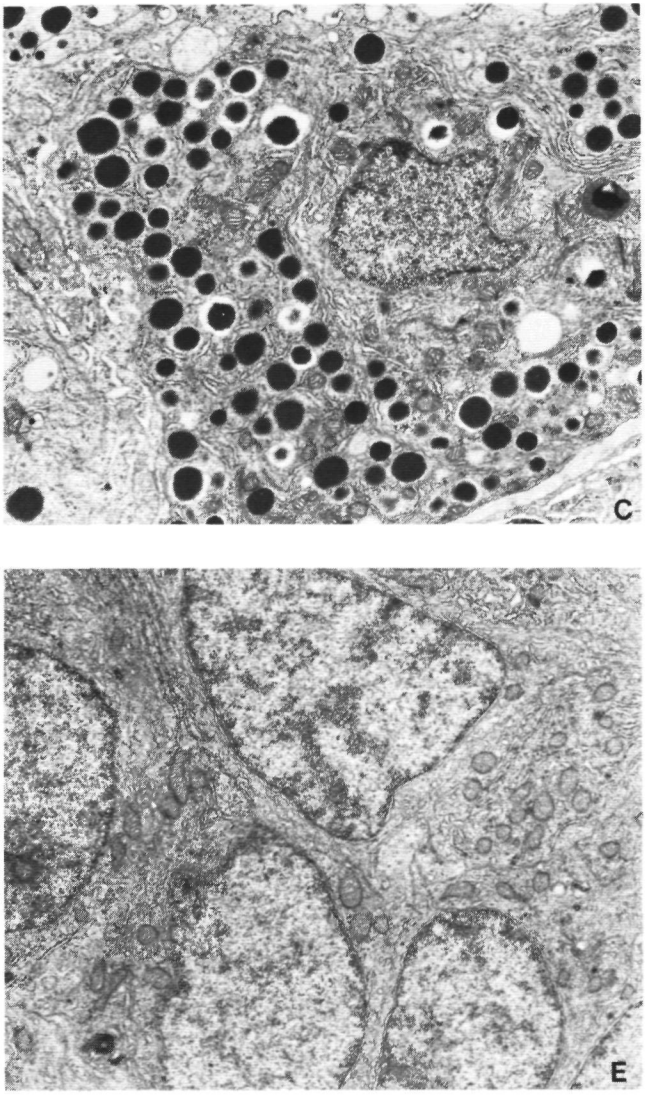

Fig 4. Electron micrographs of CS of NaCl (C) or $CaCl_2$ (E) injected eel; C: type-1 cells, with many large secretory granules; E: type-1 cells devoid of secretory granules (15000 x).

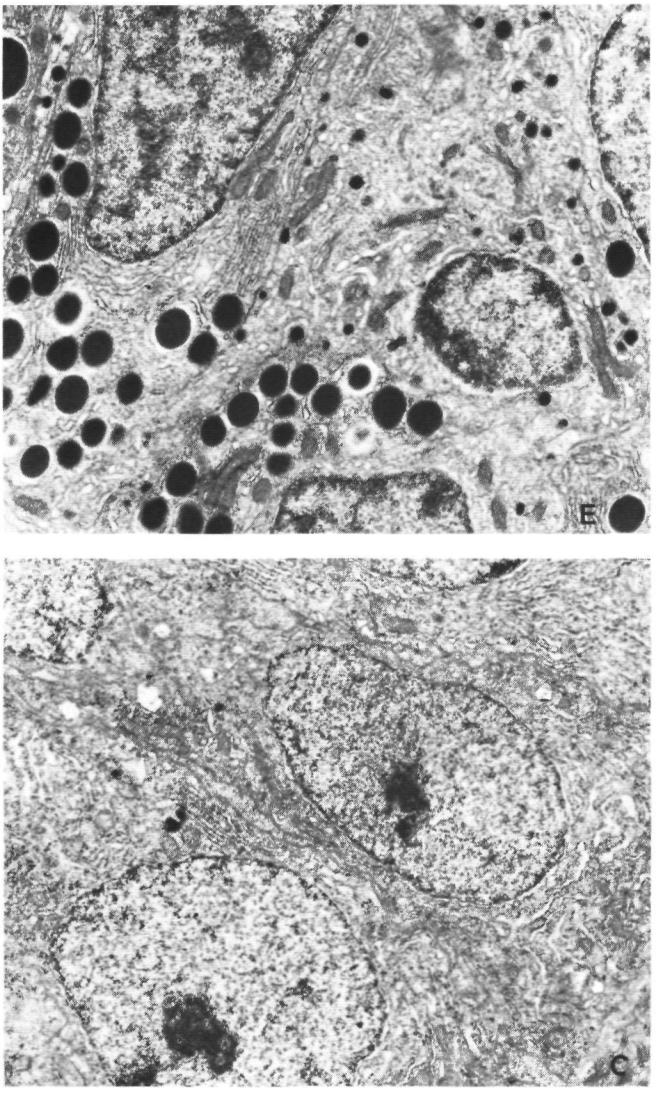

Fig 3. Electron micrographs of CS of NaCl (C) or $CaCl_2$ (E) injected trout; C: type-1 cells, with many large secretory granules; E: type-1 cells devoid of secretory granules (15000 x).

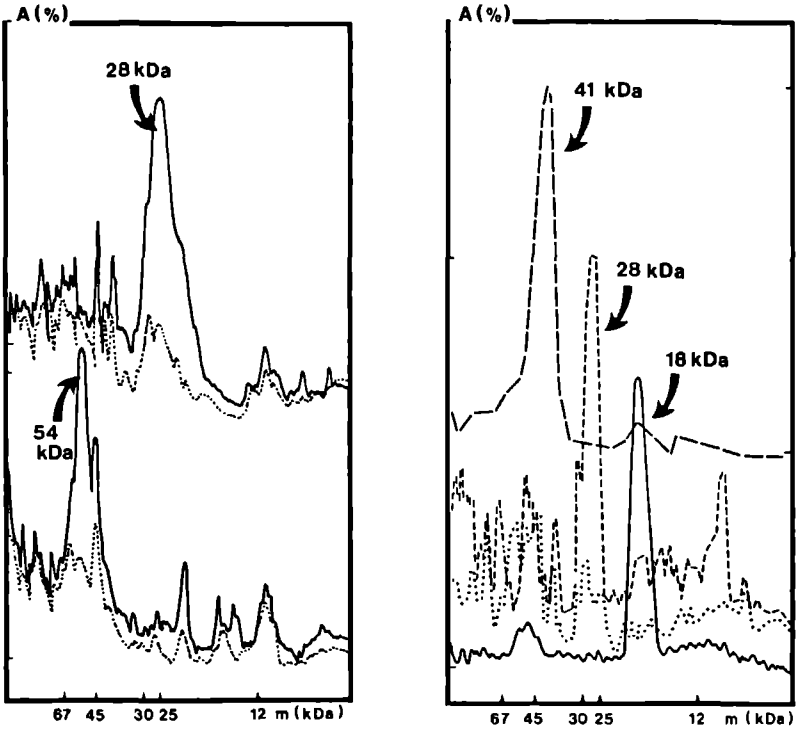

Fig 5. Densitometric scan of silverstained CS crude homogenates of NaCl (——) and $CaCl_2$ (...) injected trout after SDS-PAGE under reducing (top) and non-reducing (bottom) conditions. Absorbance is shown as % of maximum peak height (=100%). Molecular weight (*m*) of protein markers are given on the horizontal axis.

Fig 6. Densitometric scan of silverstained CS crude homogenates (---), residue (...) and isolated principle (——) after SDS-PAGE under reducing conditions (bottom). Isolated principle (— —) after SDS-PAGE under non-reducing conditions (top). Absorbance is shown as % of maximum peak height (=100%). Molecular weights (*m*) of protein markers are given on the horizontal axis.

Isolation.

Fig 6 shows a densitometric scan after SDS-PAGE of a crude CS tissue homogenate and the fractions after isolation. As shown the major constituent of the con-A binding material has an apparent molecular weight of approximately 18 kDa under reducing conditions (Fig 6 bottom). A minor contamination (with a molecular weight of aproximately

55 kDa) is observed in this fraction; this contamination is present in considerable amounts in the residue. The residue, however, does not contain the product present in the isolated fraction (18 kDa), or the products that are released from the CS after calcium injections (28 kDa). Under non-reducing conditions (Fig 6 top) the con-A binding material has a molecular weight of aproximately 41 kDa.

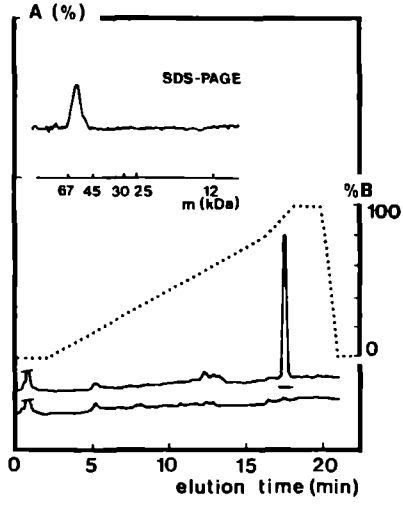

Fig 7. HPLC analysis of the con-A isolated CS 54 kDa product. Gradient (percentage of the acetonitril containing sol vent B) is shown (...). Elution time is given on the horizontal axis. Absorbance is shown in %. Insert: Densitometric scan of silverstained fraction (indica ted in the HPLC chromatogram) after SDS-PAGE under non-reducing conditions. Absorbance is shown in %. Molecular weight *m* of protein markers are given on the horizontal axis.

Protein analysis of the isolated fractions showed that from a tissue homogenate of 100 mg dry weight of CS (with a protein content of 25.3 ± 3.6 mg) 3.07 ± 0.56 mg (12%) of isolated product and 6.19 ± 0.84 mg (24%) of residue protein was recovered (n=15).

Reversed phase HPLC analyses performed to purify further the con-A binding material yielded a single symmetrical peak at 50% acetonitrile, indicating high apolarity of the product. A minor peak eluted at 35% acetonitrile, representing less than 5% of the total amount of protein. The 50% acetonitril peak has an apparent molecular weight of 54 kDa (Fig 7).

Aminoacid analysis and carbohydrate analysis.

Fig 8 shows the sequence of the first 33 N-terminal amino acids of rainbow trout isolated con-A binding product.

No differences in the N-terminal amino acid sequence were observed between the 28 kDa and the 18 kDa product. At position 7 consistently serine and glutamic acid were detected indicating a microheterogeneity in these products. Position 29 could not be identified due to a high polarity of this site. This indicates the presence of a sugar residue

linked to the amino acid chain. Carbohydrate analysis revealed that the sugar residue consisted of mannose, galactose and glucosamine, in the ratio of 1 : 1 : 2. The total sugar content made up 12% of the 54 kDa protein on a weight basis.

DISCUSSION

In this study we show the isolation of a glycoprotein from the CS of trout. This product is the hypocalcemic factor of the CS since the release of this glycoprotein from the CS is stimulated substantially and specifically upon experimentaly induced increase of the blood calcium levels. Furthermore it is shown that this product inhibits branchial calcium influx in trout wich has been suggested to be the main effect of the CS hypocalcemic hormone (39). Because of its high molecular weight we will further call this hypocalcemic glycoprotein from the CS of trout "hypocalcin". This name was proposed for a CS hypocalcemic principle with a molecular weight above 10 kDa by Pang *et al.* (10, 27), whereas the name teleocalcin was first given to a hypocalcemic principle with a molecular weight of 3 kDa isolated from the CS of salmon (25).

Hypocalcin is present in relatively large amounts in the CS of the 6 fresh water species investigated. We conclude that hypocalcin, which appears as a 54 kDa band upon SDS-PAGE, is composed of two similar subunits that appear as a 28 kDa band upon SDS-PAGE under reducing conditions. We further conclude that during isolation using con-A chromatography the C-terminal part is split off of the two subunits resulting in the appearance of an 18 kDa band upon SDS-PAGE under reducing conditions. After SDS-PAGE under non-reducing conditions this

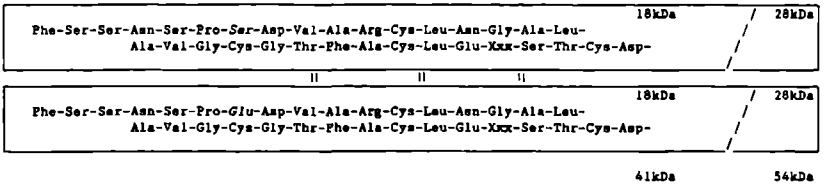

Fig 8. N-terminal amino acid sequence of trout (*Salmo gairdneri*) hypocalcin shown in a diagram of the suggested molecular form of the hormone. Position 7 shows two amino acids detected in equal amounts.

product appears as a dimer identified as a 41 kDa band. The following arguments have led us to these conclusions.

Corpuscles of Stannius of most freshwater teleost contain two different cell types: type-1 cells, which predominate in number and contain the large secretory granules, and type-2 cells, which contain the small secretory granules (see review by Wendelaar Bonga and Pang, 1986). In the literature type-1 cells are presumed to contain the hypocalcemic principle, since only type-1 cells respond to changes in external calcium concentration (38, 24, 23). In the present study we showed that an experimentally induced increase in plasma calcium was accompanied by massive release of the granules of the type-1 cells, as well as by the almost complete disappearance of the 28 kDa product from the CS. These observations lead to the conclusion that the isolated product represents the hypocalcemic principle of the CS.

The present observations on the molecular weight of 28 kDa for the hypocalcemic principle of trout and eel are in agreement with previous reports from our laboratory on the molecular weight of the CS hypocalcemic principle of the tilapia *Oreochromis mossambicus* (29). A similar 28 kDa product is present in relatively large amounts in the CS of all species investigated in the present study. This 28 kDa product is synthesized and released during *in vitro* incubations of tilapia CS, as was shown by radioactive amino acid incorporation experiments (29). Nevertheless we propose that the hypocalcemic principle of CS is a 54 kDa glycoprotein. An apparent molecular weight of 28 kDa is, however, observed upon SDS-PAGE under reducing conditions.

How does hypocalcin with an apparent molecular weight of 28 kDa, obtained by SDS-PAGE under reducing conditions, relate to the 54 kDa protein, obtained by SDS-PAGE under non-reducing conditions? We suggest that hypocalcin is composed of two subunits of similar molecular weight which dissociate under reducing conditions. The following results support this hypothesis. Under reducing conditions the 54 kDa hypocalcin appears as a single band of 28 kDa. Furthermore, the results of the amino acid sequence analysis also point to the presence of almost identical subunits. This amino acid analysis is performed under reducing conditions. This implies that amino acid sequence analysis of the 54 kDa hypocalcin in fact concerns analysis of the 28 kDa subunits. Only a single N-terminal sequence is obtained. An identical sequence is

obtained after analysis of the isolated 41 kDa hypocalcin, which actually represents the N-terminal sequence of the 18 kDa subunits obtained under reducing conditions. However, a consistent microheterogeneity was observed at position 7 of native 54 kDa as well as the isolation variant, the 41 kDa product. We suggest therefore that trout hypocalcin consists of two similar subunits that only differ at position 7. The high incidence of cysteines (4 in the N-terminal 33 aminoacids) makes the presence of disulfide bridges between the two subunits in the native molecule likely. From their recent work on the salmon hypocalcemic principle, Wagner and colleagues (22) also concluded that the salmon CS hypocalcemic principle could be composed of two subunits.

Isolation of hypocalcin yields a band of 41 kDa after SDS-PAGE under non-reducing conditions, and of 18 kDa under reducing conditions. These purified 41 and 18 kDa products are obviously related to the 54 kDa and 28 kDa products observed in freshly prepared homogenates of CS tissue. We suggest that the 18 kDa product is the N-terminal part of the 28 kDa product. The following results support this hypothesis. A tissue homogenate containing the 28 kDa hypocalcin yields an 18 kDa product upon isolation. The residue is consistently devoid of both the 28 kDa and the 18 kDa product. Furthermore, for still unknown reasons in about 1 out of 10 isolations only a 28 kDa product is isolated instead of an 18 kDa product and under non-reducing conditions only a 54 kDa product is obtained instead of a 41 kDa product. Prolonged storage of lyophilized CS tissue does result in the disappearance of the 28 kDa hypocalcin and the concomitant appearance of an 18 kDa product. Although our isolation is carried out at 0 °C we assume that endogenous protease activity converts hypocalcin from the 28 kDa to the 18 kDa product. Perhaps the most convincing evidence for the suggested relationship between the 28 and 18 kDa products is the fact that amino acid sequence analysis of both purified products reveals identical results for the first 33 N-terminal amino acids determined so far (see below). Therefore the 18 kDa product must be a N-terminal portion of the 28 kDa molecule which is split off during the isolation procedure.

Hypocalcin in the isolated fraction, obtained by con-A chromatography is slightly contaminated, as is shown by SDS-PAGE as well as by HPLC analysis. On SDS gel these contaminants (less than a

few percent on protein basis) are abundantly present in the residue. The homogeneous '50% acetonitril peak' collected by reversed phase HPLC analysis appears to be hypocalcin and to be free of contaminants. The molecular weight of 41 kDa of the trout isolated product is very close to the molecular weight of 39.3 kDa reported for the presumptive hypocalcemic factor from the CS of salmon (22). We show here, however, that the molecular weight of the 41 kDa product isolated from trout is an artefact and that the native product is a molecule of 54 kDa. It is possible that the molecular weight of 39.3 kDa reported for the hypocalcemic principle of salmon also applies to the N-terminal part of a larger molecule.

The first 33 amino acids of trout hypocalcin show remarkable overlap with the first 19 amino acids of the isolated glycoprotein from salmon reported by Wagner *et al.* (22) and the amino acid sequence of the CS principle of eel (A. Butkus, personal communication). Differences with salmon were found only on positions 3 and 18 where serine and alanine are substituted for proline and aspartic acid, respectively. For eel differences were found at the same, and five additional positions (position, trout/eel; 3, Ser/Ala; 4, Asn/Ser; 18, Ala/Glu; 21, Gly/Ser; 22, Thr/Ala; 28, Glu/Asp; 33, Asp/Asn). All substitutions could be due to single mutations, except for Ala and Gln, position 18 for trout and eel, respectively. For salmon the amino acid at position 12 has not been identified. For trout hypocalcin a cysteine is found on position 12. For salmon no cysteines were detected (22). Therefore, it may well be that salmon hypocalcemic principle has a cysteine on position 12, and that differences in the first 19 amino acids exist only at positions 3 and 18.

The amino acid on position 29 could not be determined for our isolated trout hypocalcin due to the high polarity of this position. This indicates the presence of a carbohydrate chain. Support for this hypothesis comes from the finding that position 31 is a threonine, since linkage of N-acetyl-glucosamine to aspartic acid (the most common binding) requires a sequence Asn-Xxx-Thr/Ser (40). Consequently, this would result in an Asn on position 29. For eel, an Asn is also found at this position (A. Butkus, personal comunication). Presently, however, we can not exclude the possibility of an oligosaccharide O-linked to this position, which indicates the presence of a serine or threonine.

The ratio of mannose, galactose and glucosamine (1:1:2) that we found for trout deviates from the carbohydrate composition of the hypocalcemic principle of salmon (22). We found a higher content of galactose and did not detect any glucose.

Lopez, Milet and coworkers suggested that in the CS of eels a glycoprotein is cosecreted with a proteinaceous hypocalcemic principle, and that this glycoprotein is similar to secretory protein-1 (SP-1) from the parathyroids of terrestrial vertebrates (30-32). We showed however that the isolated glycoprotein of trout is hypocalcemic and possesses PTH-like bioactivity (18, 39). In this respect our results on trout are in line with those of Wagner *et al.* (22) on salmon, who also concluded the hypocalcemic product is a glycoprotein.

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RAINBOW TROUT HYPOCALCIN STIMULATES BONE RESORPTION IN EMBRYONIC MOUSE CALVARIA *IN VITRO*, IN A PTH-LIKE FASHION

ABSTRACT.

Hypocalcin, the major hypocalcemic hormone of fish was isolated from trout CS. The bioactivity of hypocalcin was assesed in a PTH-bioassay involving bone resorption in embryonic mouse calvaria. Calcium and phosphate release and lactate production were stimulated dose-dependently by hypocalcin. On a molar basis 430 times more hypocalcin than PTH was required to obtain an effect similar to PTH in this heterologous assy. Hypocalcin did not stimulate the cAMP production either in mouse calvaria or in cultured osteoblasts. Hypocalcin resembles in this respect PTH molecules shortened or modified at the N-terminal part, that induce bone resorption without increasing cAMP levels. Since hypocalcin and PTH have comparable bioactivity in this mammalian bioassay (but also in fish bioassays) we suggest a structural similarity between both hormones. We tentatively suggest that both hormones act *via* the same receptors. Since no resemblance exists in the primary structures of PTH and hypocalcin, we hypothesize that it is the ternary structure of hypocalcin and PTH that shows similarity.

Calcium homeostasis in terrestrial vertebrates largely depends on the action of the hypercalcemic parathyroid hormone (PTH) and vitamin-D-metabolites, and of the hypocalcemic hormone calcitonin (1). Fish, like terrestrial vertebrates, regulate blood calcium levels efficiently. The endocrines involved in fish calcium metabolism differ from those of the terrestrial vertebrates. Calcium regulation in fish is dominated by the hypercalcemic action of prolactin or cortisol (2, 3) and the hypocalcemic action of the hormone from the Stannius bodies, hypocalcin (4). In fish calcitonin seems of minor importance as a calcium regulating factor (5). Removal of the corpuscles of Stannius (CS) results in an increase in the plasma calcium concentration, that may be reversed by CS implants or by injections of CS extracts or hypocalcin (Ch.3, 6-8). Hypocalcin that we isolated from trout CS (Ch.3, 8), shows substantial similarity for the N-terminal amino acid sequence with the hypocalcemic principle of eel (9) and of salmon (10).

Antigenic resemblance between hypocalcin and PTH is indicated by cross-reactivity of antisera raised against bovine PTH (bPTH) with a substance in eel blood plasma. This substance disappears from the blood after removal of the CS (stanniectomy, STX; 11). Moreover, the same antiserum to bPTH cross-reacts with a substance in the CS of the eel and this cross-reactivity disappears when the glands are stimulated to release their presumed hypocalcemic principle by experimentally induced hypercalcemia (12).

Both PTH and CS extracts decrease blood calcium levels in fish (13), and increase blood calcium levels in the rat (14). Recently, we advanced biochemical and histological evidence for a similarity in bioactivity of CS products and of PTH. Products released during *in vitro* incubation of the CS induced bone resorption in embryonic mouse calvaria *in vitro*, in a way comparable to PTH (Ch.2, 15). We suggested that both PTH and the hypocalcemic principle of the CS may act *via* the same receptor.

PTH-stimulated bone resorption depends on the activity of osteoclasts and evidence is accruing that their activity is controled *via* osteoblasts. PTH is thought to act directly on the osteoblast; the subsequent signalling from osteoblast to osteoclast may result in bone

resorption (16, 17). PTH activity may, therfore, be measured by the activity of osteoblasts. Cyclic AMP has long been considered the most important second messenger for the action of PTH on its target cells (18, 19). However, as originally suggested by Rasmussen and Tenenhouse (20), evidence is increasing that also Ca^{2+} fulfils a second messenger function (21, 22, 23, 24). Activaton of either second messenger pathway separately may result in bone resorption (21).

We report here on the effects of trout hypocalcin in a PTH bioassay involving bone resorption in embryonic mouse calvaria. We show that hypocalcin is the bone resorbing component in a CS tissue homogenate. Hypocalcin is shown to stimulate bone resorption independently of cAMP production.

MATERIALS AND METHODS

Isolation procedure.

Isolation of hypocalcin from rainbow trout (*Salmo gairdneri*) was performed by Concanavalin-A-Sepharose-4B (Con-A) affinity chromatography as described in detail elsewhere (Ch.3, 8). In short, approximately 100 mg lyophilized CS tissue (400 mg wet weight obtained from 40 kg trout) was homogenized and applied to a column. Products without affinity for Con-A are referred to as residue proteins. Material with affinity for Con-A is referred to as hypocalcin. Sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 25) in combination with a silverstaining procedure (26) was routinely performed as a purity check.

Hormone administration.

Bovine PTH (bPTH) was purchased from Sigma (TCA powder) and dissolved in 0.005 N acetic acid containing 1 % Pentex albumin. Solutions of 1 IU/µl were stored in liquid N_2. Immediately before use bPTH was diluted to the desired concentration with culture medium. No differences were observed between the bone resorbing effect of this Sigma bPTH and synthetic bPTH(1-34). Doses are expressed in IU per ml of calvarium culture medium.

Approximately 100 mg dry weight CS yielded 3.0 ± 0.3 mg purified hypocalcin and 4.5 ± 0.1 mg residue proteins. The amount of hypocalcin

that was added to the calvaria cultures is expressed in mg BSA equivalents per ml of calvarium culture medium. In accordance with the ratio of hypocalcin and residue proteins obtained after isolation (2/3), always 50 % more residue protein than hypocalcin was added in the assays.

Calvarium culture technique.

Calvaria were removed from 18-day-old mouse embryos and each calvarium was bisected. The left half of one calvarium and the right half of a second calvarium and *vice versa* were fixed in a roller tube, containing 1 ml culture medium (27). The culture medium consisted of 90 % Hanks' balanced salt solution (Hanks'BSS) and 10 % heat inactivated human serum. After 24 h of incubation at 37 °C, calvaria were removed from the incubation medium that was subsequently analyzed for calcium, phosphate and lactate.

cAMP production.

For measurements of the cAMP stimulating activity of PTH and of hypocalcin, mouse calvaria were incubated in Hanks'BSS containing 0.5 % Pentex albumin. After a 15 min incubation period at 37 °C, calvarial cAMP was extracted by ultrasonification in propanol, and measured using a phosphodiesterase binding assay (28). Results are presented in pmoles cAMP per two calvarium halves produced per 15 min.

cAMP stimulating activity was also measured in cultured chicken OB-cells. To potentiate the cAMP production the adenylatecyclase activator forskolin (10^{-7} M) was added. The phosphodiesterase inhibitor methyl-isobuthylxanthine (MIX; 0.22 mg/ml) was added to prevent breakdown of the cAMP produced. Cells were isolated and cultured as described elsewhere (27). Experiments were further performed as described above. The DNA content of the cell cultures was determined according to Karsten and Wollenberger (29). Results are expressed in pmoles cAMP per µg DNA per 15 min.

Analytical methods.

Medium total calcium content was determined with a commercial calcium-kit (Sigma). Inorganic phosphate was measured according to the method of Delsal and Manhourin (30). Combined calcium-phosphate standards (Sigma) were used as a reference. The medium lactate

concentration was measured as described by Lowry *et al.* (31) using an autoanalyser method (32). Lithium lactate (Sigma) was used as a reference. Protein content of the isolated fractions was determined with a commercial protein-kit (Biorad) using bovine serum albumin (BSA) as a reference.

Statistical analysis.

Statistical evaluation was performed by Student's *t*-test (one-tailed). Significance was accepted at $P < 0.05$. Mean values ± SEM are given.

RESULTS

Bone demineralization.

The effects of hypocalcin, the residue proteins and bPTH on calcium and phosphate release from mouse calvaria are shown in figures

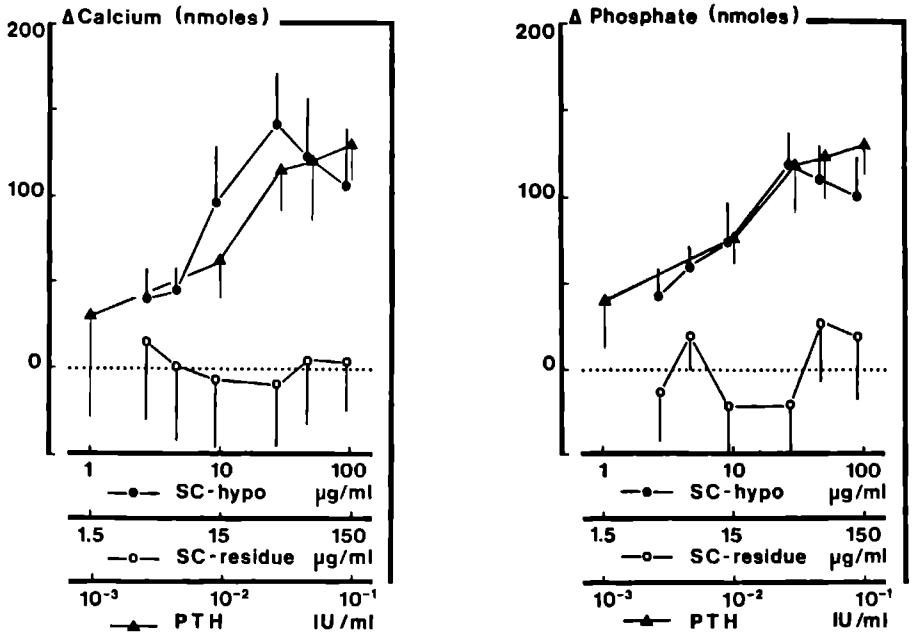

Figure 1a and 1b. Effects of hypocalcin, residue proteins and bPTH on calcium (a) and phosphate (b) release from two calvarium halves, cultured for 24 hours. Mean values ± SEM are given (hypocalcin and residue proteins n = 8; PTH n = 4).

1a and 1b. Figure 1a shows that hypocalcin and PTH stimulate calcium release dose dependently. Both hormones give a similar activation pattern. The residue proteins were without effect. Similar results were found for phosphate release (Fig 1b). Proceeding from an equipotency of 0.1 mg hypocalcin and 0.1 IU PTH in this bioassay no significant differences between the degree of stimulation by hypocalcin or by PTH for both parameters can be observed over a 2 log unit concentration range (Fig 1a & b).

A tissue homogenate of 100 mg trout CS contains bioactivity equivalent to 1 IU PTH (Ch2, 15). The amount of protein of a tissue extract of 100 mg trout CS is 4.5 ± 1.6 mg (n=16). Therefore, the specific activity of a crude tissue extract (SA_{ext}) can be expressed as 1 IU PTH activity per 4.5 mg tissue extract protein (SA_{ext}= 1/4.5 = 0.22 IU PTH/mg protein). The specific activity of purified hypocalcin (SA_{hyp}) is 1.00 IU PTH/mg protein. According to this bone resorption assay than, the purification factor for hypocalcin (SA_{ext}/SA_{hyp}) is 1.00/0.22= 4.5 .

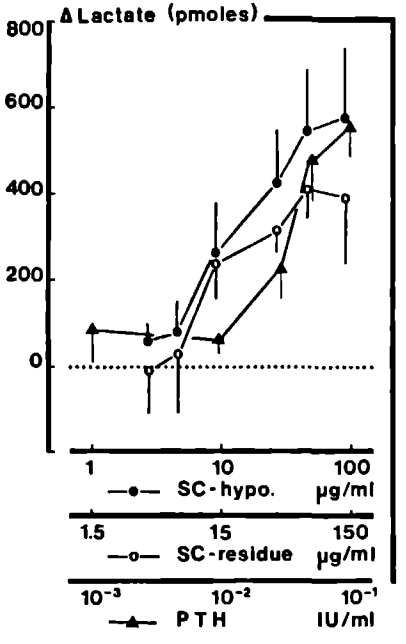

Figure 2. Effects of hypocalcin, residue proteins and bPTH on lactate production of two calvarium halves, cultured for 24 hours. Mean values ± SEM are given (hypocalcin and residue proteins n = 8; PTH n = 4).

Lactate production.

The effect of hypocalcin, residue proteins and bPTH on lactate production in mouse calvaria are shown in figure 2. Doses of hypocalcin and of PTH that give comparable responses when analyzed for calcium and phosphate release, also give a comparable stimulation of lactate production. Proceeding from the SA of hypocalcin as calculated above on the basis of calcium and phosphate release we found similar dose response curves for hypocalcin and PTH with respect to lactate production.

Residue proteins which do not stimulate release of calcium and phosphate from bone gave a significant and dose-dependent stimulation of lactate production.

cAMP production.

Table 1 shows that CS tissue extracts, residue proteins and hypocalcin have no effect on cAMP production in mouse calvaria, where PTH does stimulate cAMP production. No significant stimulation of cAMP production in the presence of forskolin was found by hypocalcin.

Table 1. Effects of CS crude tissue homogenates, hypocalcin, residue protein and PTH on cAMP production in mouse calvaria.

	(pmoles/2 calvarium halves)
control	1.4 ± 0.6 (7)
CS tissue extract (5 mg/ml)	2.1 ± 1.2 (3)
residue proteins (600 µg/ml)	2.1 ± 1.8 (6)
hypocalcin (500 µg/ml)	-1.1 ± 1.3 (9)
CS tissue ext (5 mg/ml + forsk. 10^{-7} M)	1.4 ± 3.2 (3)
PTH (.25 IU/ml)	50 ± 11 (7)*
(.5 IU/ml)	180 ± 8 (7)*

Calvaria were incubated for 10 min. Mean values ± SEM are given with the number of observations in parentheses. Asterisks indicate statistical significance.

Also, hypocalcin was unable to stimulate cAMP production in OB-like cells (both in the presence and absence of forskolin). PTH (0.05 IU), however, gave a significant stimulation of cAMP production in these cells and its effect was potentiated by forskolin (9 ± 1 and 114 ± 21 pmoles cAMP/µg DNA, respectively).

DISCUSSION

From the data presented in this paper we conclude that hypocalcin stimulates bone resorption in a PTH-like fashion. Two parameters characteristic for PTH-induced bone resorption, *viz.* calcium/phosphate release and lactate production, are stimulated dose-dependently by hypocalcin.

We previously reported that rainbow trout corpuscles of Stannius (CS) produce and secrete a product that resembles PTH in its bone resorbing ability in mouse calvaria (Ch.2, 15). The observations presented here substantiate and extend our previous report and show that a single product in a CS extract, *viz.* the glycoprotein hypocalcin, is responsible for the PTH-like bone resorbing activity.

A 4.5-fold purification calculated from the bone resorbing assay is in good agreement with a 4 fold purification that we calculated from a fish bioassay involving calcemic responses in eels (data to be published elsewhere). The low purification factor indicates that the CS store an abundance of hypocalcin. The high degree of granulation of the hypocalcin containing cells of fresh water fish (Ch.2, 15) is consistent with this notion.

The activity of 1 mg hypocalcin ($1.85 \cdot 10^{-8}$ mol) is equivalent to the activity of 1 IU PTH ($4.27 \cdot 10^{-5}$ mol). On a molar basis then 430 times more hypocalcin than PTH was necessary to obtain a similar bone resorbing effect in mouse calvaria. Although hypocalcin seems rather potent in this heterologous PTH-bioassay, PTH proved to be even more potent in an *in vivo* fish bioassay; we calculated that equimolar amounts of hypocalcin and PTH have a similar hypocalcemic effect in eel (data to be published elsewhere). Apparently, both hypocalcin and PTH may evoke physiological responses in a mammalian as well as in a fish bioassay. We tentatively conclude therefore, that both calciotropic hormones may activate PTH-dependent as well as hypocalcin-dependent targets.

Hypocalcin is able to increase lactate production in mouse calvaria. Lactate production may serve as a parameter for PTH induced bone resorption since PTH induced bone resorption is always coupled to lactate production (27, 33). PTH fragments reported to induce bone resorption (21) also induce lactate production (34). Residue proteins, which were not able to induce calcium and phosphate release, were also able to stimulate lactate production in a dose dependent way. This suggests that residue proteins contain aa factor which is capable to enhance calvarial metabolism without increasing bone resorption. These observations corroborate our previous results where we showed that a CS tissue extract contains two products that may stimulate lactate production: a factor that stimulates bone resorption and a factor which

does not stimulate bone resorption (Ch.2, 15). Although PTH induced bone resorption is always coupled to lactate production, lactate production is not necessarily coupled to bone resorption (33). Therefore, lactate production *per se* is not an exclusive parameter for bone resorbing activity.

Although cAMP is reported to be an important second messenger for the bone resorbing activity of PTH, hypocalcin was, in contrast to PTH and despite its PTH-like bone resorbing effects, not able to increase cAMP production. However, it has been demonstrated that the bone resorbing effect of PTH is not fully dependent on cAMP as a second messenger. PTH fragments modified or shortened at the N-terminus induce bone resorption without increasing cAMP levels (21). We therefore suggest that hypocalcin to some extent resembles PTH but lacks, in agreement with the forementioned PTH fragments, that part of the molecule that is essential for cAMP production.

On the basis of the similarities in bioactivity of hypocalcin and PTH (N-terminal shortened fragments) in the PTH bioassay as reported in this paper and in a previous paper (Ch.2, 15), we suggested that both hormones act *via* the same receptor site. In this case hypocalcin should stimulate bone resorption *via* changes in cytosolic Ca^{2+}, similarl to what has been shown for shortened PTH fragments (24). Unfortunately, tests on the effects of hypocalcin on Ca^{2+} influx in OB-like cells using the Quin2 fluorescence method (24) appeared not to be feasible because of the high autofluorescence of hypocalcin. Our former observation, however, that hypocalcin (CS tissue extract) increase the number of osteoclasts in mouse calvaria (Ch.2, 15) -the increase in number of osteoclasts is suggeted to be mediated by Ca^{2+} as a second messenger (35)- gives indirect evidence that hypocalcin may induces Ca^{2+} influx in bone cells. In this respect hypocalcin can be a useful tool to further investigate the two binding sites of the PTH receptor and the relative contribution of both second messengers in the PTH induced bone resorbing activity (35).

Several factors like prostaglandins (36, 37), phorbolesters (38) and PTH-like tumor factors (39) may induce bone resorption without activating the PTH receptor. One could argue then that the bone resorbing activity of hypocalcin also could result from the activation of a pathway different from the one activated by PTH. However, several

notions are in favor of a common receptor-second messenger pathway activated by hypocalcin and PTH. There is a striking similarity in the effects induced by both hypocalcin and PTH with respect to the bone resorbing activity (including the increase in the number of osteoclasts; Ch.2, 15). We have also shown, by testing maximum stimulating concentrations of hypocalcin containing CS tissue extracts and PTH together, that additive effects of both hormones for bone resorption may be excluded (Ch.2, 15). Hypocalcin and the forementioned PTH fragments have in common that they induce bone resorption without a detectable increase in cAMP production. Furthermore, the similarity between PTH and hypocalcin is not restricted to PTH-like bone resorbing activity *in vitro*. CS tissue extracts have been reported to be hypercalcemic in rats (14); conversely, PTH and hypocalcin have been reported to be hypocalcemic in intact fish (40). We have recently confirmed that PTH and hypocalcin exert hypocalcemic effects in stanniectomized eels (41).

The similarity in bioactivity, however, seems not congruous with the fact that there is no similarity in amino acid sequence between hypocalcin and PTH. Although immuno-crossreactivity has been reported between some PTH antisera and the products released by the CS (11, 12), the amino acid sequence of hypocalcin (Ch.3, 8-10) shows no similarity with PTH (or any other known peptide). Furthermore, whereas PTH is a peptide, hypocalcin is a glycoprotein possibly composed of two subunits (Ch.3, 8). We postulate therefore that these hormones partially resemble each other, not in their primary structure but in their three dimensional configuration. This would be a plausible explanation for the immunological similarities as well as for the similarities in bioactivity of these hormones in homologous and heterologous bioassays.

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Chapter 5

HYPOCALCEMIC ACTIVITY OF TROUT AND EEL HYPOCALCIN AND OF BOVINE PTH IN STANNIECTOMIZED EELS

ABSTRACT

In this paper we show that hypocalcin isolated from trout and eel CS as well as bovine PTH (bPTH) exert hypocalcemic effects when injected intra-arterially in hypercalcemic stanniectomized (STX) eels. Upon injection of both hypocalcins we observe a decrease in blood ionic calcium which fully accounts for the decrease in total calcium. Injections of bPTH also result in a decrease of total and ionic calcium concentrations of the blood. The decrease in ionic calcium upon injection of bPTH, however, does not fully account for the decrease in total calcium concentration of the blood. Nevertheless, hypocalcin and bPTH show striking similarity in bioactivity in stanniectomized eels. Some similarity in three dimensional structure of both hormones may underlie this phenomenon.

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Corpuscles of Stannius (CS) are small endocrine glands characteristic for bony fish. Eels have one pair of CS, that is located ventrocaudally to the kidney. More than the calcitonin secreting ultimobranchial glands, the CS have been suggested to be involved in hypocalcemic control in fish (1-3). Removal of the glands (stanniectomy, STX) results in an increase in the blood calcium concentration, whereas injections of CS homogenates or reimplantations of CS correct STX-induced hypercalcemia (1, 4, 5).

In the literature a variety of products has been reported to be secreted by the CS (3, 6-8). However, the main bioactive secretory product of the CS seems to be a glycoprotein (Ch.3, 9, 10). This glycoprotein has been isolated from salmon CS by Wagner *et al.* (9) and named teleocalcin, whereas we recently have isolated the glycoprotein from trout (Ch.3, 10). We have called the principle hypocalcin, the name originally proposed by Pang *et al.* (11) for the active factor of the CS. The products from salmon and trout show only small differences in molecular weight and substantial similarity in the N-terminal amino acid sequence (Ch.3, 9, 10). The N-terminal sequence derived from DNA base analysis of the principle of the Australian eel is also similar to that of trout and salmon (12). Wagner *et al.* (9) have shown that the isolated salmon principle inhibits ^{45}Ca uptake in juvenile rainbow trout. We showed that trout hypocalcin inhibits branchial Ca^{2+} influx in adult trout (Ch.8, 13).

Imunological resemblance has been shown between a CS principle and the parathyroid hormone (PTH) of the terrestrial vertebrates (14-16) and it has been suggested that the CS are homologues of the parathyroid glands (17). CS tissue extracts and PTH show similar activities in mammals and fish (Ch.2, 14, 16, 17, 18). Wendelaar Bonga *et al.* (19) have shown hypocalcemic activity of bPTH(1-34) in low calcium acclimated killifish and tilapia. On the other hand, the latter authors have questioned the homology of CS and parathyroid glands on the basis

of reported differences in embryological origin (3). Furthermore, Butkus and colleagues (12) have shown that there is no similarity in the predicted amino acid sequence of eel hypocalcin and PTH.

In the present study we have isolated a 54 kDa glycoprotein from eel CS. We have tested the ability of this principle, trout hypocalcin, and of bPTH on their ability to decrease plasma calcium levels in STX eels. From a physiological point of view, ionic calcium is more important than the total calcium concentration of the blood (20, 21). We have therefore monitored the ionic as well as the total blood calcium concentration after hormone injections.

MATERIALS AND METHODS

Animals.

Trout (*Salmo gairdneri*), used for the collection of the CS, with a body weight between 200 and 300 g and were obtained from a commercial trout farm (Nijmegen, The Netherlands).

European eels (*Anguilla anguilla*), of 230-290 g body weight, were obtained from several commercial fish mongers. They were collected only in the summer; the fish were sexually immature. The eels were acclimated to Nijmegen tapwater, for at least 3 weeks but no longer than 6 weeks, in 500 l opaque well aerated fibre-glass tanks. Water was filtered by recirculation over charcoal (1000 l/h) and refreshed with a constant inflow of tapwater (100 l/h). Total calcium concentration of the water was 0.65 ± 0.5 mM. Water temperature was between 10 an 12 °C.

Analytical techniques.

Plasma total calcium was determined using a commercial calcium reagent kit (Sigma). Ionized calcium and pH of whole blood were measured within 30 min after sampling in an automated ionic calcium analyzer (ICA-1, Radiometer; 22). Protein was measured using a commercial protein reagent kit (Biorad). Osmolality was measured in a Roebling micro-osmometer.

Isolation of hypocalcin.

Hypocalcin was isolated as described recently (Ch.3, 10) using concanavalin-A (con-A) affinity chromatography. Material bound to con-A consisted for trout as well as eel of a glycoprotein with an apparent molecular weight of 41 kDa (hypocalcin). Hypocalcin fractions proved to be pure for at least 95 % on protein basis. The material without affinity to con-A is referred to as residue proteins and was almost devoid of hypocalcin as judged by sodiumdodecylsulphate-polyacrylamide gelelectrophoresis (SDS-PAGE) (23) and bioassay (Ch.8, 13).

Stanniectomy and cannulation.

Eels were anaesthetized in sodium bicarbonate buffered (pH 7.8) ethylaminobenzoate (MS222, 2 g/l). During operation that lasted up to 10 min fish were kept wet and CS were removed from the kidney via a small incision through the body wall and the kidney capsule (2 cm long sagittal incision, at the position of the anus; 24). Muscle and skin were sutured. The fish were allowed to recover in 200 l well aerated tanks with a constant flow of fresh tapwater (100 l/h). The CS obtained from these fish were collected on solid CO_2 and used for isolation of hypocalcin as described above.

Ten to twelve days after this operation fish were anaesthetized again and the pneumogastric artery was cannulated according to procedures described by Chester Jones *et al.* (25). They were kept in individual opaque polyvinyl tubes with a diameter of 5 cm, perfused with running, well aerated tapwater (50 l/h). Cannulas were conducted out of these tubes through a longitudinal slot in the tubes and were flexibly fixed above water level. This set up allowed free movement of the fish and stress free blood sampling. Fish were allowed to recover for at least 2 days. The cannulas were used for injection and blood sampling. Between these procedures the cannulas were filled with a sodium chloride solution (0.6 %) containing polyvinyl pyrrolidone (0.12 g/ml) and sodium heparin (500 U/ml), and closed with a metal pin.

Experimental procedures.

Six groups of fish were injected at day 15 after STX, with either trout hypocalcin, eel hypocalcin, trout residue proteins, eel residue proteins, bPTH (Sigma) or vehicle (0.9% saline solution). These

injections were immediately followed by an injection of a 500 µl blood sample which was previously drawn from the fish.

The amounts of protein injected per 100 g fish were 50 µg hypocalcin and 80 µg residue proteins; these doses equal the amount of material obtained from 6 fish. Bovine PTH was injected in a concentration of 20 IU per 100 g fish.

Blood samples were subsequently taken for three days. A blood sample of 100 µl was drawn to clean the cannula. Subsequently, a 250 µl blood sample was drawn with a 1 ml syringe containing 10 µl of Ca^{2+}-heparin (Radiometer). After determination of ionic calcium and pH the remaining 140 µl blood was centrifuged (1 min at 9000*g*). Plasma was used for determination of the total calcium, osmolality and heamatocrit concentration. Two blood samples were taken before injection and eight samples over a three day period after injection. The pH, osmolality and heamatocrit were measured to check the viability of the eels during experimentation.

Statistical evaluation.

Mean values ± SEM are given. For statistical evaluation Mann-Whitney *U* test (one tailed) was used. Significance was accepted at $P < 0.05$. For statistical evaluation of differences between experimental and control injected groups and differences between the decrease in total and ionic calcium, values obtained at the same time after STX and after injection were compared.

RESULTS

Stanniectomy of the eels resulted after 15 days in a plasma total calcium concentration of 5.71 ± 0.21 mM and a blood ionic calcium concentration of 3.21 ± 0.16 mM (n = 50). Before injection, there was no significant difference in total or ionic calcium concentration between the different treated groups (vehicle treated control group, two groups treated with hypocalcin, two with residue proteins and one group with bPTH).

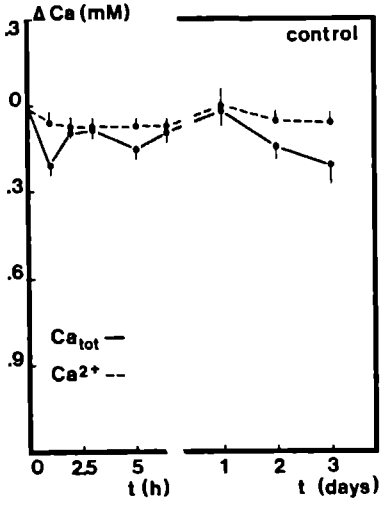


Fig. 1. The effect of NaCl injection (control) on plasma total calcium (Ca_{tot}, ——) and blood ionic calcium (Ca^{2+}, - -) in 15 day STX eels. Means ± SEM are given. At t=0: Ca_{tot} = 5.58 ± 0.22 mM, Ca^{2+} = 3.12 ± 0.22 mM; n=15.

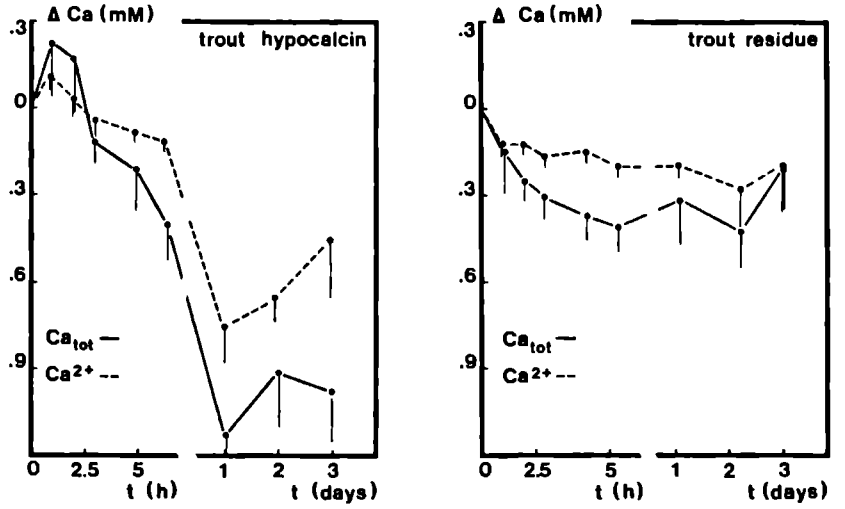
After injection of NaCl (controls), no significant changes in either of the two calcium concentrations were observed over the three day period the samples were taken (Figure 1). The maximum decrease was 0.08 ± 0.04 and 0.22 ± 0.05 mM for ionic and total calcium, respectively.

Injections with trout and eel hypocalcin resulted in a decrease of total and ionic calcium (Figures 2a and 3a, respectively). When injected in the same dose the reduction of plasma calcium levels was similar for both preparations. The blood calcium lowering effect started within a few hours after injection and reached its maximum after 24 hours. Although after 24 hours the calcium concentrations were tending upwards, the difference with the controls was still statistically significant three days after injection.

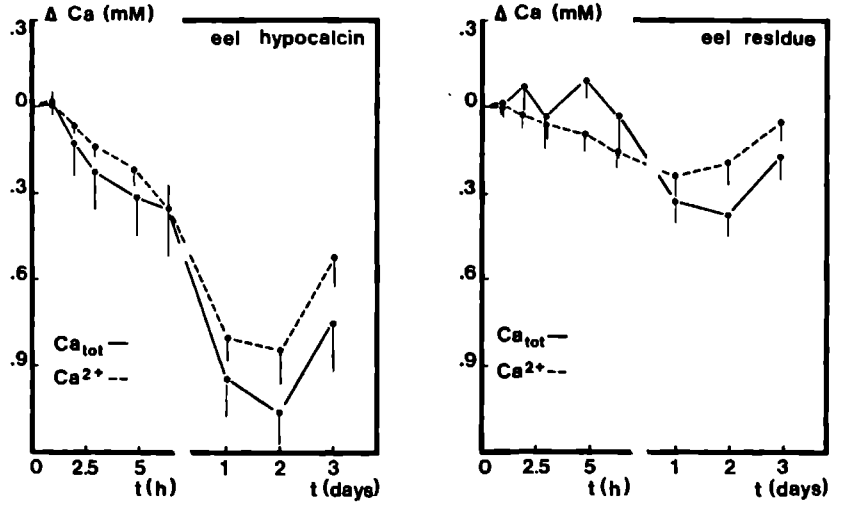
The decrease in plasma total calcium and the decrease in blood ionic calcium were similar. The differences between the reduction in ionic and total calcium were not statistically significant.

Injections with residue proteins resulted neither in a significant change of total calcium, nor of ionic calcium (Figures. 2b and 3b).

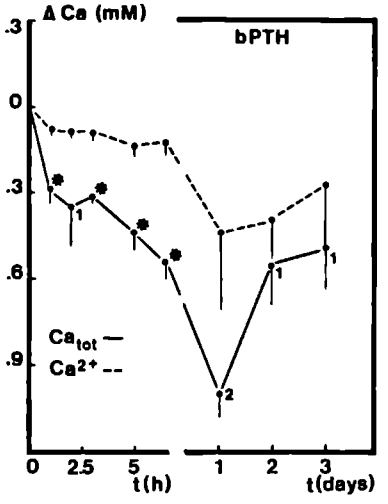
Injection with bPTH resulted in a decrease of the plasma total calcium as well as the ionic blood calcium concentration (Figure 4). The decrease in total calcium concentration, however, was more pronounced than, and statistically different from, the decrease in ionic calcium.



Figs. 2a and 2b. The effect of trout hypocalcin (a) and trout CS residue proteins (b) on plasma total calcium (Ca_{tot}, ——) and blood ionic calcium (Ca^{2+}, - -) in 15 day STX eels. Means ± SEM are given. At t=0: Ca_{tot} = 4.62 ± 0.52 mM, Ca^{2+} = 3.06 ± 0.37 mM for hypocalcin (n=5); Ca_{tot} = 6.26 ± 0.22 mM, Ca^{2+} = 3.07 ± 0.13 mM for residue proteins (n=7).



Figs. 3a and 3b. The effect of eel hypocalcin and CS eel residue proteins on plasma total calcium (Ca_{tot}, ——) and blood ionic calcium (Ca^{2+}, - -) in 15 day STX eels. Means ± SEM are given. At t=0: Ca_{tot} = 5.88 ± 0.28 mM, Ca^{2+} = 3.58 ± 0.20 mM for hypocalcin (n=9); Ca_{tot} = 5.59 ± 0.37 mM, Ca^{2+} = 3.28 ± 0.27 mM for residue proteins (n=10). After residue injection the effect on total calcium is only significant at t=24 h ($0.04 < P < 0.05$).



The pH was measured for all blood samples and did not change during the experiments (pH = 7.9 ± 0.1). Osmolality and hematocrit were determined for 5 animals which were all diferently treated. No significant changes during the experiment were observed for blood hematocrit (35 ± 12 %) or plasma osmolality (289 ± 6 mOsmol/kg).

Fig. 4. The effect of bPTH (Sigma crude extract) on plasma total calcium (Ca_{tot}, ——) and blood ionic calcium (Ca^{2+}, - -) in 15 day STX eels. Means ± SEM are given. At t=0: Ca_{tot} = 6.47 ± 0.49 mM, Ca^{2+} = 2.99 ± 0.41 mM (n=4). Decrease in ionic calcium did not differ statistically from the decrease in controls. Asterisks indicate statistically significant differences between the decrease in ionic and total caclium concentrations; 1: $P < 0.10$, 2: $P < 0.06$).

DISCUSSION

In this paper we show that the glycoprotein isolated from trout and eel CS, which we tentatively have named hypocalcin (Ch.3, 10), induces a decrease of plasma total calcium in hypercalcemic STX eels. Since the CS residue proteins do not show a significant calcium reducing activity in contrast to the isolated product, we conclude that the isolated glycoprotein may indeed be equated with the hypocalcemic principle of the CS for which Pang *et al.* (11) have proposed the name hypocalcin. The decrease in calcium concentration after a single injection with hypocalcin was maximal one day after injection and persists for at least 3 days. This is in line with observations on eels that it takes at least some days before plasma calcium levels become elevated after removal of the CS (*e.g.* 5, 26).

The glycoproteins we isolated from trout and eel CS have an apparent molecular weight of 41 kDa. In a previous study we have found

the same molecular weight for trout hypocalcin (Ch.3, 10). This value is in good agreement with the apparent molecular weight (39 kDa) of the hypocalcemic principle isolated from salmon CS (9). However, as we concluded in a recent paper (Ch.3, 10), the isolated 41 kDa product probably forms the N-terminal part of a 54 kDa product. The latter was considered to be the native hypocalcin molecule.

Hypocalcin isolated from CS of both trout and eel and injected in the same dose in the eel, results in an effect similar for both hypocalcins, which indicates a low species specificity of hypocalcin. This supports observations of Pang *et al.* (11, 27) who have shown a decrease of blood calcium concentrations in low calcium acclimated killifish after injection with CS crude tissue homogenates of a variety of fish species.

Infusion of both hypocalcins results in a decrease in blood ionic calcium which fully accounts for the decrease in total calcium. This contrasts with the suggestion of Bailey and Fenwick (28) that the CS stimulate the binding of ionic calcium to plasma proteins, without affecting total plasma calcium concentration. Our results show that upon hypocalcin treatment of hypercalcemic STX eels normal ionic calcium levels are restored, without changes in the other blood calcium fractions. We therefore conclude that the hypocalcin regulates the blood ionic calcium fraction. This is probably effected by inhibition of gill Ca^{2+} influx. We have shown that hypocalcin injections reduce branchial Ca^{2+} influx without affecting Ca^{2+} efflux, which results in a net loss of calcium *via* the gills (Ch.8, 13).

From a physiological point of view, the blood ionic calcium concentration may be more important than the blood total calcium concentration. This has been extensively shown for many higher vertebrates. For instance, McLean & Hastings (20) have elegantly demonstrated that a frog heart reacts as a Ca^{2+} selective electrode. There is, however, a limited number of reports on ionic blood calcium homeostasis in fish. Recently Andreasen (21) has reported that the blood ionic calcium level in trout is kept constant in different stress situations. In intact eels CS extracts caused a decrease of the ionic calcium fraction, without effecting other parameters (28).

Infusion of bPTH also results in a decrease of total and ionic calcium concentrations of the blood. The dose used (20 IU/100 g fish)

results in a response not significantly different from the effect induced by 50 µg hypocalcin/100 g body fish. This means that on a molecular basis about equal amounts of PTH and hypocalcin are required to obtain a similar calcium lowering effect. The presence of parathyroid glands has never been reported for fish, whereas CS have never been observed in terrestrial vertebrates. The effects of PTH in teleost fish has attracted attention since 1958, when Budde tested PTH in the guppy and showed effects on bone mineralization (29). Effects of CS extracts on terrestrial vertebrates have been reported by Milet *et al.* (14), who have shown an increased calcium release from rat bones *in vitro*. In 1980 Milet et al. have reported on the immunological resemblance between PTH and a principle from the CS (15). This inspired further study of the similarity between PTH and hypocalcin. Wendelaar Bonga *et al.* (19) have recently shown a hypocalcemic effect of PTH in killifish and tilapia adapted to low calcium water. We have reported that CS tissue homogenates as well as the isolated glycoprotein induce bone resorption in embryonic mouse calvaria *in vitro* (Ch.2, 18). These observations may point to a homology between the CS and the parathyroid glands as has been suggested by Milet, Lopez and coworkers (15, 17). However, although both hypocalcin and PTH reduce plasma calcium levels in fish, there is some difference between the effect of hypocalcin treatment and PTH treatment. Whereas the reduction of plasma calcium by hypocalcin is mainly effected by a change in ionic calcium, bPTH induces a decrease in ionic calcium that does not fully account for the decrease in total calcium. Apparently, both ionic and protein bound calcium are decreased by PTH. This suggests that hypocalcin and PTH may act, possibly partially, via different mechanisms. Hypocalcin is reported to inhibit gill Ca^{2+} influx without affecting Ca^{2+} efflux, which results in a net loss of calcium *via* the gills (Ch.8, 13). In addition to the inhibition of gill Ca^{2+} influx (15), PTH may act on bone mineralization in fish (29).

The supposed homology between PTH and hypocalcin has been doubted recently. Wendelaar Bonga *et al.* (19) reviewed reports indicating that the parathyroids and the CS have a different embryological origin. Furthermore, the N-terminal amino acid sequence for salmon and trout hypocalcin (Ch.3, 9, 10) as well as the predicted total amino acid sequence of eel hypocalcin as reported by Buskut *et al.* (12) show no

similarity with the aminoacid sequence of PTH. This is also at variance with the supposed homology between both products. Nevertheless, there is a striking similarity in bioactivity between PTH and hypocalcin, in mammalian as well as fish studies. Some similarity in the three dimensional structure of both hormones may underlie this phenomenon.

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MECHANISMS INVOLVED IN HYPOCALCEMIC REGULATION

(Part II)

INTRODUCTION

As stated before, corpuscles of Stannius (CS) are without question involved in hypocalcemic control. The mechanisms which underlie this hypocalcemic control, however, are ill-defined. In terrestrial vertebrates calcium homeostasis of the blood is mainly controlled by the regulation of intestinal calcium uptake and by the buffering activity of the bone. Calcium regulation in fish is particularly interesting because of the intense interaction of these organisms with their aquatic environment. Calcium regulation in fish is probably fully dependent on the regulation of the exchange of calcium with the water. Fish gills contain very efficient mechanisms for the regulation of calcium exchange between the ambient water and the body fluid, which probably are hormonally controlled (1). The aim of this study was to investigate the function of the corpuscles of Stannius (CS) in the hormonal control of this exchange. The involvement of the CS in this process is indicated by studies of Milet and Fenwick (2, 3). The results of these studies were contradictory, however. The isolation of hypocalcin from the CS as described in part I enables us to study more specifically the mechanisms involved in hypocalcemic control in fish. The following considerations were the basis for this study.

Fontaine (1964) was the first to show that the CS are involved in calcium regulation (4). Removal of the glands (stanniectomie; STX) from the eel resulted in a very pronounced increase of plasma calcium levels. Hypercalcemia after STX has since been reported for several other species, *e.g.* killifish, goldfish, sticklebacks and tilapia, although for technical reasons most of the experiments reported were carried out on eels (5-8). Re-implantation of CS or injections of CS tissue homogenates reversed STX induced hypercalcemia (11-12).

The source of calcium responsible for the hypercalcemia after STX was unclear. STX of eels adapted to low calcium water resulted in a reduced hypercalcemia, whereas fish adapted to acalcemic water did not

develop hypercalcemia (*e.g.* 6, 8). This indicates that the ambient water is the source of calcium responsible for this effect. Contradictory results, however, have been reported and bone has also been suggested to be the source (13, 14). The observation that CS homogenates reverse STX induced hypercalcemia does not necessarily imply that the CS have a hypocalcemic function in intact fish. True hypocalcemic activity of CS extracts in fish, *i.e.* a decrease of plasma calcium levels in intact fish, has been reported only twice. In these experiments hypocalcemia could, however, only be evoked with CS extracts in fish adapted to low calcium water and fed a low calcium diet (8, 15). Histological examination of the CS of fish adapted to high or low calcium environments revealed that the glands are more active in calcium rich water (*e.g.* 16-17). This is a further indication that the glands are involved in the regulation of the calcium exchange between water and fish.

The kidney, gut and gills have been suggested to be involved in the control of calcium handling between water and fish. A decrease of urinary calcium concentrations following STX has been reported (18), although contradictory results have also been shown (13). Injections of CS extracts decreased intestinal calcium absorbtion *via* the gut (19). The latter effect, however, may be of minor importance for total calcium uptake from the environment, since calcium uptake mainly takes place *via* the gills (1). For this reason the gills have been considered the most important target for hypocalcin (1). Gills from STX eels displayed a considerably higher net calcium uptake than the gills of sham operated eels (2, 20). In another study, gill arches from 1 week STX eels showed an increased influx of calcium and a decreased efflux of calcium (3). Furthermore, it was shown that perfusion of gill arches with CS extracts resulted in a decrease of the calcium influx and an increase of the calcium efflux. The calcium lowering effect of CS tissue extracts injected in STX eels has therefore been suggested to originate from a decreased calcium uptake from the water (3). However, under control conditions as wel as STX conditions the net flux of calcium was negative, which makes these experiments difficult to interpret.

Considering the gills as the major target organ for hypocalcin, what mechanisms then are involved in calcium handling of the gills and how may these mechanisms operate? It has been reported that the chloride cells in the branchial epithelium are responsible for the calcium uptake by the gills (21). A model for gill calcium handling in fresh water fish has been originally postulated in our laboratory for tilapia (22). More recently this model has been extended to trout (23). It proposes that the Ca^{2+} efflux is passive and paracellular, and controlled by the trans-epithelial potential (TEP) and the chemical gradient for Ca^{2+} between extracellular fluid and the ambient water. Ca^{2+} influx on the other hand is thought to be transcellular. It involves a passive entry of calcium at the apical membrane of the chloride cells from water to cytosol *via* an electro-chemical gradient. Calcium transition from cytosol to the blood is achieved by an ATP driven calcium pump located at the basolateral membrane. On the basis of this model hypocalcemic control in freshwater fish can be effected by increasing Ca^{2+} efflux or by decreasing Ca^{2+} influx across the branchial epithelium. An increase of Ca^{2+} efflux can be realized by increasing the permeability of the paracellular branchial route of the epithelium. Decrease of Ca^{2+} influx may involve an inhibition of Ca^{2+} entry into the chloride cells and/or an inhibition of the Ca^{2+} pump.

In this part of my thesis the participation of the CS and more specifically of hypocalcin in the hypocalcemic regulation in fish is studied. It is shown that hypocalcemia after STX is a prolonged phenomenon indicating that the CS are the only endocrine glands involved in hypocalcemic control. Results obtained with injections of CS extracts in STX eels show that the CS are involved in the regulation of ionic calcium (Ch.7). Furthermore, it is shown that injection of hypocalcin in trout inhibits specifically and acutely Ca^{2+} influx across the gills without effecting Ca^{2+} efflux; a hypothesis for the mechanism of action of hypocalcin at the cellular level is presented that is based on the above described model (Ch.8). Experimentally increased blood calcium levels evoke the release of endogenous hypocalcin and inhibits branchial Ca^{2+} influx which is further evidence for the hypocalcemic function of the CS (Ch.9).

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Chapter 7

EFFECTS OF STANNIECTOMY AND INJECTIONS OF STANNIUS CORPUSCLE EXTRACTS ON TOTAL AND IONIC CALCIUM LEVELS IN THE EEL

ABSTRACT

Removal of the corpuscles of Stannius (stanniectomy, STX) from freshwater eels increased the total and ionic calcium concentrations in the blood. The increase in ionic calcium starts later and is relatively smaller than that the increase of total calcium which leads to a decrease of the ratio of the blood ionic over plasma total calcium concentrations (Ca^{2+}/Ca_{tot}). Apparently, the increase in ionic calcium was limited by enhanced Ca^{2+} binding to plasma proteins. Notwithstanding, blood ionic calcium levels increased more than two fould. Hypercalcemia reached a maximum between 18 and 22 days after STX. Although calcium levels tended to decrease thereafter, they still were significantly elevated 40 days after STX. Intra-arterially injected crude tissue extracts of CS of eel, trout or salmon in eels 15 days after STX caused a decrease of ionic and total calcium levels. The reduction of the total calcium concentration was almost completely due to a reduction of ionic calcium. We conclude that the CS produce the main hypocalcemic hormone in eel which is involved in the regulation of the ionic calcium level of the blood.

In a variety of teleost species removal of the CS (stanniectomy; STX) resulted in a rise of plasma total calcium (*e.g.* 1-8). In several of these studies, the subsequent hypercalcemia was found to be transient; normocalcemia was observed again within 5 to 8 weeks after STX (2, 9, 10). The development of hypercalcemia was dependent on the water calcium concentration (*e.g.* 6, 9, 11, 12). In acalcemic water no rise in plasma calcium concentrations was observed after STX (13). Injection of CS homogenates (1, 4, 7, 14) or reimplantation of the corpuscles (11, 13) reversed STX induced hypercalcemia.

Beyond doubt the CS are of major importance for the regulation of plasma calcium; yet, their effect on plasma ionic calcium has received little attention. Using a frog heart, McLean and Hastings (15) were the first to show that it is the ionic fraction of plasma calcium which is physiologically important. For trout it has recently been shown that ionic calcium levels are kept constant under different stress situations (16). Reports on ionic calcium levels in fish, however, are limited. In humans, plasma total calcium can be divided in a protein-bound (38%) and an ultrafiltrable or dialytic fraction (62%). The latter fraction consists of complexed calcium and ionic calcium which represent 10% and 52% of plasma total calcium, respectively (17). The amount of calcium associated with the blood cells as compared to the amount of calcium in the plasma is negligible (16) and, therefore, the concentrations of the calcium fractions in the plasma reflect the concentrations of the respective fractions in whole blood.

According to Chan and Chester Jones (18), the plasma ionic calcium fraction in eels is rather constant and comparable with the ionic calcium fraction found in mammals; in the freshwater eel about 54% of the total plasma calcium is in the ionic form. The concentrations of plasma ionic calcium reported range from 1.3 to 1.6 mM Ca^{2+} (3, 5, 18). This relatively large variation may well be due to the techniques available at that time to measure ionic calcium rather than reflect true biological variation.

Only two reports evaluated plasma ionic calcium concentrations after STX. Chan (3) gave provisional evidence for an increase in plasma

ionic calcium in 14 days STX Japanese eel and Fenwick (5) reported an increase of ionic calcium concentrations two weeks after STX in the North American eel. Chan (3) concluded that the initial rise in plasma total calcium following STX was mainly due to a rise of plasma ionic calcium. In these experiments ionic calcium levels, but not total calcium levels, returned to normal approximately 2 weeks after STX. The decrease in ionic calcium level was suggested to result from an increase in plasma calcium binding proteins, since total calcium concentrations stayed elevated up to 5 weeks after STX. Fenwick (5), on the other hand, reported that the increase in total calcium after STX exceeded the increase in ionic calcium; both calcium fractions were still elevated 3 weeks after STX. Recent reports on plasma ionic calcium levels after STX and subsequent replacement therapy are lacking. The techniques to measure blood ionic calcium concentrations have recently been improved, and therefore we re-evaluate in this report the effects of STX on blood ionic and total calcium levels in the eel. Furthermore, we show the effects of injections of CS extracts in STX eels on blood ionic and total calcium concentrations. Parameters crucial for the evaluation of ionic Ca^{2+} concentrations in the blood, *viz.* blood pH and plasma protein content were determined as well.

MATERIALS AND METHODS

Animals.

Immature European eels *(Anguilla anguilla)*, ranging in body weight from 200 to 350 g, were obtained from commercial fish dealers in the Netherlands. Upon arrival in the laboratory the fish were kept in 300 l aerated tanks supplied with running Nijmegen city tapwater (total calcium between 0.6 and 0.7 mM). The fish were not fed. The temperature of the water was between 11 and 13 °C. Fish were acclimated for at least 3 weeks but for no longer than 6 weeks before experimentation.

Freshwater rainbow trout (*Salmo gairdneri*), weighing around 250 g were obtained from a commercial trout farm (Beek, The Netherlands) and were used for collecting CS.

Lyophylized CS of coho salmon (*Oncorhynchus kisutch*) were a generous gift from Prof. J.C. Fenwick (University of Ottawa, Canada).

Surgery.

For surgery the eels were anaesthetized in sodium-bicarbonate buffered ethylaminobenzoate (MS 222, 2.5 g/l, pH 7.8). Eels were stanniectomized or sham-operated as described by Leloup-Hatey (19). Muscle and skin were sutured carefully and the fish were allowed to recover in 100 l tanks containing aerated running tapwater. The CS of the STX fish were collected on solid CO_2 for subsequent preparation of CS extracts.

The pneumogastric artery was cannulated as described by Chester Jones and colleagues (20). Cannulated eels were kept in individual opaque polyvinyl cylinders supplied with running tapwater as described before (21). The set-up allowed free movement of the eels and relatively stress-free blood sampling.

Preparation of CS extracts.

Lyophylized CS of eel, trout or salmon were homogenized in ice cold saline (0.6% sodium chloride), with a Potter type homogenizer. The supernatant obtained after centrifugation of the homogenate at 9000 *x g* (5 min) was used for injection. For eel and salmon an extract of 1,0 mg dry weight CS material was injected per 100g fish; for trout an extract of 0.75 mg dry weight CS material per 100 g fish was used. Injection volume was 200 µl/100 g fish. Saline injections served as control.

Experimental protocol.

Stanniectomy. Serial blood samples of the eels were taken over a period of 17 days maximally. To cover a period of 40 days, three groups of 16 eels each were used to study the effect of stanniectomy in time. From each group half of the eels was stanniectomized and the other half sham-operated. The three groups were cannulated 2 days before, and 11 and 26 days after STX or sham-operation, respectively. Blood samples of 200 µl were taken once a day using a 1 ml syringe containing 5 µl of calcium heparin (Radiometer); sampling started 2 days after cannulation and lasted 15, 17 and 12 days for each group, respectively. In a separate experiment a single blood sample was taken by cardiac puncture from 6 fish, 40 days after STX, to evaluate possible effects of the cannulation and blood sampling procedure on plasma calcium concentrations.

Hormone injections. CS extract of eel, trout and salmon was injected *via* the cannula. Before injection a 500 µl blood sample was taken, of which 200 µl was used for plasma analyses. The remaining 300 µl was re-injected immediately following the injection of the CS extract. Eight 200 µl blood samples, were taken with a 1 ml syringe containing 5 µl calcium heparin (Radiometer) over a 3 days period after injection.

Analytical techniques.

Blood samples were analyzed for ionic calcium and pH within 15 min after sampling using an automated ionic calcium analyzer (ICA-1, Radiometer). Blood was sampled from fish kept in water of 12 °C while ionic calcium was determined at 37 °C (the ICA-1 have a fixed measurement temperature). The overall temperature effect on Ca^{2+} levels in human blood is an increase of ± 0.002 mM/°C (22). Assuming a change in ionic calcium of 0.002 mM/°C in eels, we calculated that the increase in temperature from 12 to 37 °C results in an overestimation of blood ionic calcium with 0.05 mM.

The blood remaining after ionic calcium analysis was centrifuged (1 min at 9000 *x g*) and plasma was analyzed for total calcium using a commercial calcium-kit (Sigma). Combined calcium-phosphate standards (Sigma) were used as reference.

Plasma osmolality was measured with a micro-osmometer (Roebling) using distilled water and a 300 mOsmol/kg standard (Biorad) as reference.

Plasma protein content was determined using a commercial calcium-kit (Biorad). Bovine serum albumin protein standard (Biorad) was used as a reference.

Statistics.

The data are presented as means ± SEM. The Mann Whitney *U*-test (one-tailed) was used for statistical evaluation. Significance was accepted at $P < 0.05$. To evaluate effects of STX on blood parameters, values of STX-eels were compared with the corresponding values of the sham-operated eels. To evaluate the effects of CS extract injections, values of the CS extract injected eels were compared with the

corresponding values of the saline injected eels. Effects are presented as the change in plasma total calcium or in blood ionic calcium levels relative to these calcium levels before injection.

RESULTS

The changes in blood ionic and plasma total calcium after STX are shown in Figure 1. The average ratio of ionic calcium over total calcium (Ca^{2+}/Ca_{tot}) in eels stanniectomized for 15 days or longer was 0.49 ± 0.03. When compared to controls, STX caused an increase in total as well as in ionic calcium. The increase in plasma total calcium was evident within the first day after injection and reached a maximum of

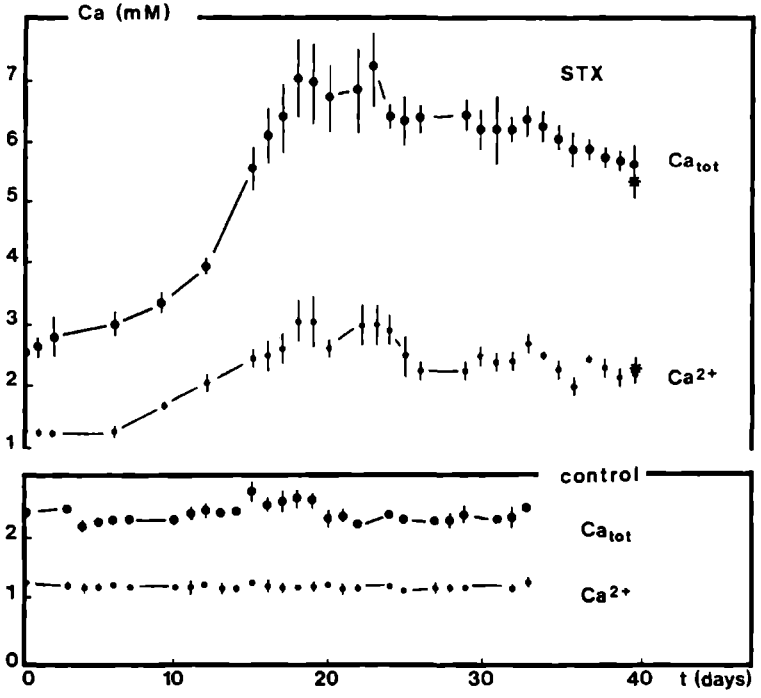

Fig 1. Effect of stanniectomy (STX) and sham operation (control) on the blood total (—) and ionic calcium (--) concentration of eels. Values are presented as means ± SEM (n = 8). Asterisks indicate blood total and ionic calcium concentration determined in non-cannulated eels from a single blood sample taken by cardiac puncture.

approximately 7 mM between 18 and 22 days after STX. Blood ionic calcium levels became elevated only 6 days after STX and reached a maximum of approximately 3 mM within the same time period. The rise in total calcium surpassed the rise in ionic calcium. This resulted in a significant decrease of the ratio Ca^{2+}/Ca_{tot} to 0.40 ± 0.04. Total and ionic calcium concentrations tended to decrease 22 days after STX, but were still significantly higher than those measured in sham-operated eels.

At day 40 after STX, blood ionic and plasma total calcium concentrations did not differ significantly from the corresponding calcium concentrations determined in blood samples taken by cardiac puncture from non-cannulated and non-sampled STX eels.

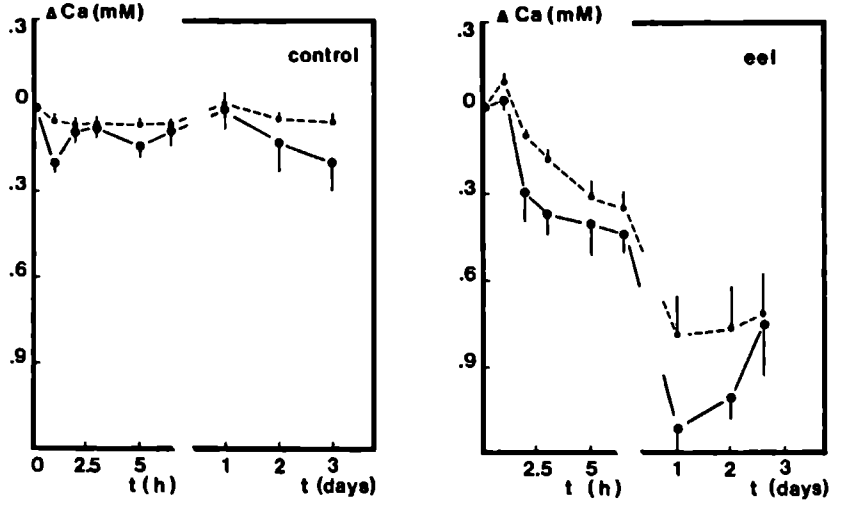

Fig 2a. Effect of saline injections on blood total (—) and ionic calcium (--) concentrations in eels 15 days after stanniectomy. Values presented as means ± SEM (n = 18) are shown as differences compared to pre-injection levels.

Fig 2b. Effect of eel CS extract injection on blood total (—) and ionic (--) calcium concentrations in cannulated eels 15 days after STX. Values, presented as means ± SEM (n = 11) are shown as differences compared with pre-injection values.

Saline injections did not result in a significant change of either of the two blood calcium concentrations (Fig 2a). Injections of CS extracts of eel, of trout and of salmon in 15 days STX eels resulted in a significant decrease of plasma total and blood ionic calcium levels (Fig 2b,c,d). At the time of injection, 15 days after STX, total and ionic plasma calcium levels did not differ significantly between the four treatment groups. The average pre-injection values were 5.77 ± 0.20 for total and 3.03 ± 0.15 mM for ionic calcium. A maximum decrease of total and of ionic calcium concentration was observed 1 day after injection, whereupon calcium concentrations returned to pre-injection levels. The decrease in ionic calcium accounted completely for the decrease in total calcium. No significant differences were observed between the decrease in total calcium and the decrease in ionic calcium.

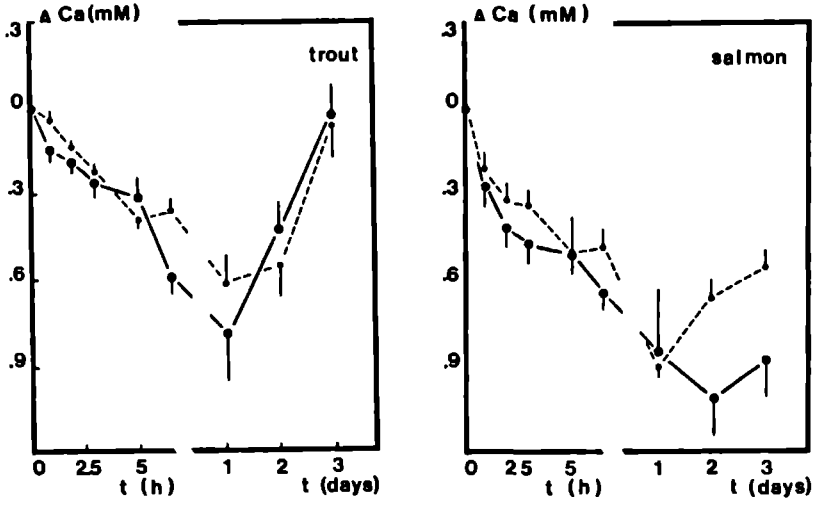

Fig 2c and d. Effect of injections of trout (b) and salmon (c) CS extracts on blood total (—) and ionic (--) calcium concentration in cannulated eels 15 days after STX. Values, presented as means ± SEM (n = 11), are shown as differences compared with pre-injection values.

None of the treatments, either STX or replacement therapy effected hematocrit, blood pH, plasma protein content or plasma osmolality. The blood pH varied between 7.85 and 7.95, plasma protein concentrations was 40 ± 5 mg/ml, and plasma osmolality was 289 ± 7 mOsmol/kg (n = 7).

DISCUSSION

Removal of the CS from fresh water eels caused a marked increase in plasma total calcium levels. In this respect our data confirm earlier reports on eels and other teleosts (1-5, 8, 23, 24). The hypercalcemia observed after STX is extreme and a unique and interesting phenomenon. To our knowledge no other calciotropic endocrine gland has been described of which the removal results in such an extreme response.

In the present experiments plasma calcium levels were measured over a 40 days period after STX. Plasma calcium levels tended to decrease but remained elevated until day 40. Blood samples from non-cannulated STX eels confirmed that the persistence of the hypercalcemia was not due to the cannulation or disturbance caused by the repetitive sampling procedure. Our data contrast with those of others who reported that the elevated plasma calcium levels in STX eels decrease significantly or even become normal again (*e.g.* 3, 10, 25). This discrepancy may be caused by differences in experimental conditions (*e.g.* the calcium level of the water). Unfortunately, in most reports on STX the time span of the measurements was too short or too few calcium measurements were done to evaluate restoration of blood calcium levels.

How does STX influence blood ionic calcium levels? We observed that the blood ionic calcium level, in contrast to the plasma total calcium concentration, did not rise within the first days after STX. Furthermore, after STX the ratio of ionic calcium over total calcium (Ca^{2+}/Ca_{tot}) decreased by about 20%: the protein bound calcium concentration increased by about 0.7 mM. Apparently most of the Ca^{2+} entering the fish during the first days after STX is buffered by enhanced Ca^{2+} binding to plasma proteins. This interpretation is in

line with observations of Chan (3), who found that ionic calcium returned to normal before total plasma calcium concentrations decreased. He suggested that an increase in Ca^{2+} binding to plasma protein, brought about by an increase in the amount of plasma proteins, was responsible for the decreased ratio of ionic calcium to total calcium. However, we did not observe a significant change in the plasma protein concentration. When hypercalcemia has reached its maximum, the concentration of calcium bound to plasma proteins (40 mg/ml) has increased from 1.25 mM ($[Ca_{tot}]$=2.5mM; $[Ca^{2+}]$=1.25mM) to 4 mM ($[Ca_{tot}]$=7mM; $[Ca^{2+}]$=3mM). This implies the binding of about 0.10 mmol calcium/g protein, which corresponds to the calcium binding of plasma proteins reported for humans (0.12 mmol calcium/g protein; 27).

In the present experiments changes in blood pH never exceeded 0.1 pH unit. It is important to keep in mind that changes in blood pH may effect the ionic calcium concentration. In human whole blood an increase of 0.1 pH unit (in the pH range 7.0 - 8.3) results in a decrease of ionic calcium of approximately 0.06 mM (28, 29). Assuming that the same relation holds for eel blood we conclude that pH related effects on blood ionic calcium concentrations are negligible in our experiments.

Injection of homogenates of CS from eel, trout and salmon in STX eels resulted in a decrease in total calcium concentration which corroborates previous reports on the injections of CS extracts in STX fish (*e.g.* 1, 7) and is very similar to the decrease we observed after injections of purified trout and eel hypocalcin (Ch.5). The present data show that the decrease in total calcium was completely accounted for by the decrease in ionic calcium. This clearly indicates that the CS are involved in regulation of ionic calcium. If hypocalcin mainly controls plasma ionic calcium one could argue that removal of the CS should initially have caused an increase in ionic calcium fraction. After STX, only the protein bound fraction of calcium had increased and later on the increase in protein bound calcium surpassed that in plasma ionic calcium. We suggest, however, that this phenomenon reflects the calcium buffering capacity of plasma proteins, which appears to be large and may buffer ionic calcium levels under normal conditions. This ionic calcium binding capacity of plasma protein may obscure the effects on blood ionic calcium levels after removal of the CS.

It has been suggested that an increased release of calcitonin by the ultimobranchial glands may redress STX induced hypercalcemia (10, 24), although no evidence has been presented so far. According to previous reports (3, 9, 10) it takes at least 5 weeks before plasma calcium levels return to normal whereas in our experiments plasma calcium levels remained elevated for at least 6 weeks. Assuming that the ultimobranchial glands are indeed involved in hypocalcemic regulation in fish, we may conclude that the capacity of calcitonin to decrease plasma calcium levels in STX eels is very small compared to that of the CS hormone. The present data show that injection of CS extracts in STX eels decrease plasma calcium levels within hours. Transplants of CS in STX fish correct the hypercalcemia within days (13). Furthermore, their is no consensus in the literature on the function of calcitonin in calcium regulation in fish. In most experiments, calcitonin exerts only minor hypocalcemic effects, if any (26). We conclude therefore that the ultimobranchial glands are of minor importance for hypocalcemic regulation and that no other hypocalcemic hormone than that of the CS effectively controles plasma calcium levels in eel.

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HYPOCALCIN FROM STANNIUS CORPUSCLES INHIBITS GILL CALCIUM UPTAKE IN TROUT

ABSTRACT

Bidirectional whole body flux and branchial calcium influx were measured in freshwater rainbow trout. Intra-arterial injections of homogenates of Stannius corpuscles (CS) as well as of a 54 kDa isolated product (hypocalcin) exerted an inhibitory effect on whole body Ca^{2+} influx, but did not effect Ca^{2+} efflux. Hypocalcin was more effective in reducing Ca^{2+} influx in trout acclimated to low-calcium freshwater than in fish from normal-calcium water. We conclude that the isolated product (hypocalcin) represents the hypocalcemic principle of the CS. Similar doses of hypocalcin caused quantitatively similar decreases in Ca^{2+} influx *in vivo* and in the isolated perfused head preparation. This indicates that the gill forms the principle target for hypocalcin in trout. The branchial transepithelial potential did not change during hormone treatments. A possible mechanism of hypocalcin action is suggested.

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Teleost fish such as trout are able to regulate their blood calcium levels efficiently (2, 11). It is well known that fish absorb calcium directly from the water and that this uptake route can account for their entire calcium requirement (5). The gill is the major site of calcium exchange between water and fish and, therefore, the gill plays a predominant role in the maintenance of the fish's calcium balance (4, 5, 11). One may reason, then, that the gill will be the primary target for calciotropic hormones in fish (4).

An important calciotropic factor in fish is the hypocalcemic principle from the corpuscles of Stannius (CS) (see ref. 22 for a recent review). The mechanisms of action of the CS principle are less well known, although the gill has been anticipated as one of its target organs (11). In isolated perfused eel gill preparations from stanniectomized fish, an increase in Ca^{2+} uptake was demonstrated (3, 16). Perfusion of CS tissue homogenate caused a reduction of Ca^{2+} uptake (10, 17).

Recently, we have reported on the isolation and identity of a 54 kDa product of trout CS (Ch.3, 9). Injection of this principle in hypercalcemic stanniectomized eels results in a decrease of plasma calcium levels (unpublished results). We have called this principle hypocalcin, the name proposed by Pang *et al.* (12) for the hypocalcemic principle of the CS. Here we will demonstrate that hypocalcin exerts an inhibitory control on the branchial calcium influx in trout that may account for the hypocalcemic action of this hormone. Since it was shown that the hypocalcemic action of the CS principle is notable only when it is injected into fish adapted to low-calcium water (21), we have examined its effect on whole body Ca^{2+} in- and efflux in fish acclimated to both normal- and low-calcium freshwater. To establish the relative importance of the gill for whole body Ca^{2+} flux, Ca^{2+} influx also was determined in an isolated head-gill preparation.

Animals

Trout (*Salmo gairdneri*) used for collection of CS and isolation of hypocalcin were purchased from a commercial trout farm (The Netherlands). The fish used for (*in vitro*) branchial flux experiments (173 ± 6 g; n=19) were acclimated to Nijmegen tap water as described in detail by Verbost *et al.* (18).

Trout used for (*in vivo*) whole body Ca^{2+} flux experiments (183 ± 4 g (SEM) body weight; n=158) were obtained from a commercial dealer (Ontario, Canada). The fish were acclimated for at least 3 weeks to dechlorinated Ottawa tap water containing (mM): Ca^{2+} (0.5), Cl^- (0.1), Na^+ (0.1), K^+ (0.02); this water is referred to as normal-calcium water. A group of fish were acclimated to low-calcium water, for at least 3 weeks. Low calcium water was prepared by titrating demineralized water with $CaCl_2.2H_2O$ to achieve a final Ca^{2+} concentration of 0.04 mM. The Cl^-, Na^+ and K^+ concentrations were adjusted to the levels of normal-calcium water. Our set up allowed a constant flow of low-, or normal-calcium water through 1000 liter fibre glass tanks. Water temperature was 15 ± 3 °C and the water pH varied between 6.3 and 7.4. The photoperiod was 12 hours of light alternating with 12 h of darkness. Fish were fed daily to satiation with commercial trout pellets.

Analytical methods.

Water and plasma total calcium contents were determined with a commercial calcium-kit (Sigma). Combined calcium-phosphate standards (Sigma) were used as reference. Protein content was measured with a commercial reagent kit (BioRad) using bovine serum albumin (BioRad) as a reference. ^{45}Ca contents of water samples and of plasma samples were determined after addition of Scinti Verse II (Fisher) by liquid scintillation analysis. ^{47}Ca contents of water and plasma samples were determined by gamma-ray spectrometry.

Isolation of hypocalcin.

Hypocalcin was isolated as we recently described (Ch.3, 9), using concanavalin-A (con-A) affinity chromatography. Material bound to con-A

consisted of at least 98 % of a 54 kDa glycoprotein, which we named hypocalcin. The material without affinity to con-A (crude CS homogenate minus hypocalcin) is referred to as residue proteins and was devoid of hypocalcin as judged by SDS-gelelectrophoresis.

Determination of whole body Ca^{2+} flux.

Catheterisation. Fish were anaesthetised in sodium bicarbonate buffered (pH 7.4) ethylaminobenzoate (MS222; 0.1 g/liter). During operations, that lasted up to 10 min, fish were kept wet and the gills were irrigated with MS222-containing water. Dorsal aortic catheterisation was subsequently performed using standard techniques. Catheters were filled with slightly modified Cortland saline containing 10 U/ml ammonium heparin and were closed with a stainless steel pin. Fish were allowed to recover on the operating table by irrigation of the gills with plain water. Next, they were left for at least 24 hours prior to experimentation in individual flux boxes supplied with running normal- or low-calcium water.

Experimental procedures. Whole body flux rates were determined using catheterised fish kept in 3 liter opaque Perspex boxes. At the start of an influx experiment, water flow through the boxes was discontinued and ^{45}Ca was added to the water (5×10^5 Bq/liter; ICN Canada). Efficient mixing of the ^{45}Ca with the water was assured by vigorous aeration of the flux boxes. After a 4 hour exposure period the fish were killed with an overdose of MS222 (0.8 g/l). Next, the fish were rinsed in tap water (1 min) and transferred for 3 min to water containing 10 mM $CaCl_2$ to exchange ^{45}Ca adsorbed to the integumental surfaces. To determine total body ^{45}Ca activity, fish were microwave-cooked (1.5 min) and homogenized in a commercial blender with 65% body weight of distilled water. Quintuple homogenate samples (approximately 0.5 g weighed to the nearest 3 decimals) were dissolved at 60 °C overnight in 2 ml of tissue solubiliser (NCS; Amersham). Subsequently, the samples were neutralized with glacial acetic acid; 5 ml of distilled water was added and the ^{45}Ca activity determined.

For efflux experiments, fish were injected (*via* their catheter) with 2.22×10^6 Bq of ^{45}Ca in 0.6 % NaCl (vol = 1 ml), 24 h before the start of the flux period. Ten min before the start of an experiment, water flow through the boxes was discontinued and ^{45}Ca appearance rate in the water monitored (see below). After a flux period of 4 hours, the

^{45}Ca-containing water was replaced by tracer free water until ^{45}Ca reached background levels. Subsequently, a second 4 h flux period was performed. The first flux measurement served to establish individual control values; the second flux period was used to determine the effects of vehicle or hormone treatment.

Flux determinations were performed according to Flik *et al.* (5) and Perry & Wood (14). For influx measurements we assumed a constant ^{45}Ca accumulation rate by the fish for at least 4 h. Flik *et al.* (5) showed that in tilapia, the ^{45}Ca accumulation rate under comparable conditions was constant for that time period. Water ^{45}Ca specific activity (SA) did not decrease significantly over the 4 h flux period. For efflux determinations a constant ^{45}Ca tracer appearance rate in the water for at least 4 h (measurements were performed every 15 min) for control as well as hormone-treated fish was observed (r_o = 0.999, significance of the slope $P < 0.01$). Renal calcium excretion was either constant or, when intermittent, did not influence water ^{45}Ca specific activity since no fluctuations in ^{45}Ca appearance rate in the water were observed. A 24 h period of internal ^{45}Ca distribution was sufficient to ensure a constant plasma ^{45}Ca SA (< 5 % change) during the flux period.

Product administration. All injections (200 µl) were given intra-arterially *via* the catheter at t=0 of the flux period. Three types of treatments were given: a) crude CS tissue homogenates, b) hypocalcin, and c) residue proteins. The amounts of protein injected per 100 g fish were 235 µg CS tissue homogenate, 50 µg hypocalcin and 100 µg residue; these doses equal the amount of material obtained from 3.75 (crude tissue homogenate) and 6.5 fish of similar body weight, respectively. Cortland saline injections served as controls.

Blood and water sampling. For influx as well as efflux measurements 500 µl blood samples were withdrawn *via* the catheter immediately before hormone injection (t=0) and upon completion of the flux experiment (t=4 h). During influx experiments 5 ml water samples were taken at t=15 min and at the end of the flux period (t=4 h). For efflux determinations, water samples were taken at the start, middle, and at the end of the flux period (t= 0, 2 and 4 h, respectively). Blood samples were centrifuged in heparinized 1.5 ml tubes. Plasma (100 µl) and water (5 ml) samples were analysed in triplicate for ^{45}Ca

specific activity (SA).

Calculations. Influx of Ca^{2+} was calculated from the total ^{45}Ca activity of the fish after 4 h exposure to ^{45}Ca-containing water and the mean SA of the water, and was normalized to fish weight. Ca^{2+} efflux was calculated from the slope of the time curves of the ^{45}Ca appearance in the water and the mean ^{45}Ca SA of the plasma and was normalized to fish weight. Flux is expressed in µmol per h per kg fish.

Determination of branchial Ca^{2+} influx.

Experimental procedures. To determine branchial Ca^{2+} influx the isolated head preparation was used as described by Payan & Matty (13), with some modifications according to Perry & Wood (14). Procedures for Ca^{2+} influx calculation, and determination of transepithelial potential (TEP) have been described in detail recently by Verbost *et al.* (18). At the start of the experiment ^{47}Ca (2.9×10^6 Bq/litre) was added to the water. The Ringer solution was well aerated throughout the experiments.

Product administration. CS crude tissue homogenates, hypocalcin, and residue proteins were added to the Ringer used to perfuse the gills. A 30 min perfusion with plain Ringers to determine control influx was followed by a 15 min perfusion with hormone containing Ringer, using separate containers connected by a three way valve. Hormone was added in concentrations of 50 µg/100 ml Ringer for the isolated product, 100 µg/100 ml Ringer for residue proteins and 150 µg/100 ml Ringer for crude tissue homogenate.

Perfusate and water sampling. For Ca^{2+} influx the ^{47}Ca appearance rate in the perfusate collected from the dorsal aorta was determined (the dorsal aortic outflow represents 90-95% of the total ^{47}Ca influx; 14). Perfusate samples (100 µl) were taken every 1 min for the first 10 min and every 5 min thereafter until t=30 min, followed by 10 one min intervals and a final 45 min sample. Water samples of 1 ml were taken at the same time. These samples were used to determine the water ^{47}Ca SA.

Calculations. Ca^{2+} influx was calculated on the basis of tracer appearance in the perfusate and the water ^{47}Ca SA. Control influx was calculated at t=25-30 min; influx after hormone treatment was calculated at t=40-45 min.

Statistical analysis.

Data are presented as mean values ± SEM. For statistical evaluation the Mann-Whitney *U*-test, one tailed, was used. Significance was accepted at $P < 0.05$.

RESULTS

Whole body Ca^{2+} flux.

Controls. Ca^{2+} influx values in untreated fish show only small variations for normal-calcium acclimated fish as well as for low-calcium acclimated fish. No significant variation of Ca^{2+} influx is observed over the two month period in which the experiments were performed.

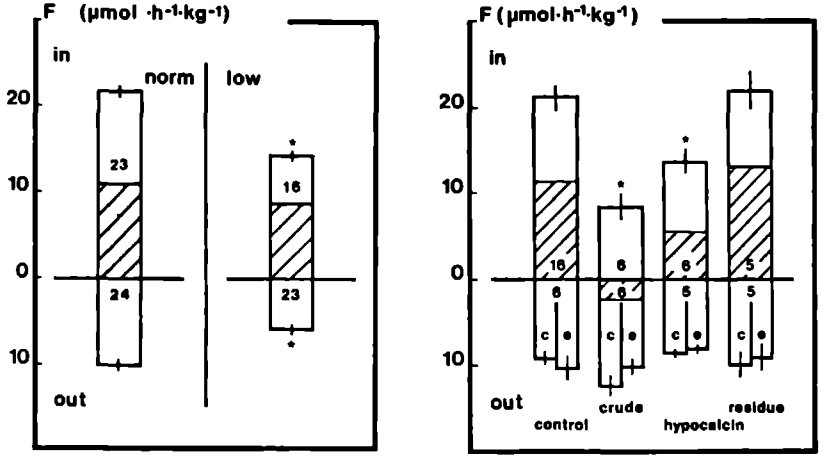

Fig 1. Calcium influx (in), Ca^{2+} efflux (out) and Ca^{2+} net flux (hatched bars) for normal- and low-calcium acclimated fish. Mean values ± SEM are given; number within the bars indicate n. Asterisks indicate significant difference from normal-calcium acclimated fish.

Fig 2. Effects of CS crude homogenate, hypocalcin, and residue proteins on Ca^{2+} influx (in) and Ca^{2+} efflux (out) for normal-calcium acclimated fish. For efflux: control flux (c; fish without treatment) is given in the left side of the bars; experimental flux (e; fish after treatment) is indicated on the right sides of the bars. Hatched bars indicate Ca^{2+} net flux. Mean values ± SEM are given; numbers within the bars indicate n. Asterisks indicate significant difference from the control. .sp

Low calcium acclimation. In Fig. 1 influx, efflux and net flux of calcium are shown for low-calcium acclimated fish and these are compared with flux values for normal-calcium acclimated fish. Under normal and low-calcium conditions net flux of calcium does not differ significantly, although both influx and efflux are lower in low-calcium acclimated trout.

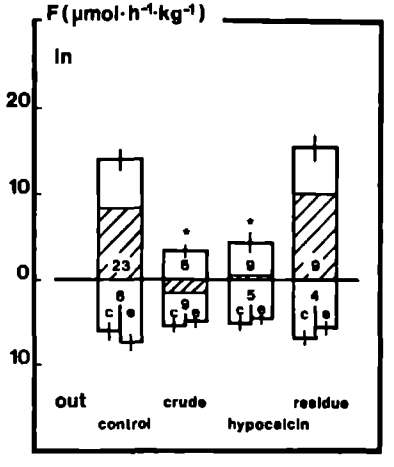

Table 1 Effects of CS crude homogenate, hypocalcin and residue proteins on branchial Ca^{2+} influx (F_{in}).

	F_{in} (%)	
control	92.7 ± 0.4 (4)	
crude	80.6 ± 3.1 (4)	*
hypocalcin	79.3 ± 2.7 (7)	**
residue	92.7 ± 4.1 (4)	

Mean values ± SEM are given in % (n) of pre-hormone Ca^{2+} influx (100 % came to 7.28 ± 1.13 µmol per hour per kg). Asterisks indicate statistical significance (* $P < 0.014$; ** $P < 0.003$).

Fig 3. Effects of CS crude homogenate, hypocalcin, and residue proteins on Ca^{2+} influx (in) and Ca^{2+} efflux (out) for low calcium acclimated fish. For efflux: control flux (c; fish without treatment) is given in the left side of the left side of the bars; experimental flux (e; fish after treatment) is indicated on the right side of the bars. Hatched bars indicate Ca^{2+} net flux. Mean values ± SEM are given; numbers within the bars indicate n. Asterisks indicate significant difference from the control.

Hormone effects. Figs. 2 and 3 show the effects of the various treatments on the Ca^{2+} in- and efflux values in normal and low-calcium acclimated fish compared to the controls (saline injected). Crude CS tissue homogenates and the isolated hypocalcin inhibit calcium influx in both groups. Similar doses of hypocalcin yield significantly greater inhibition in low-calcium acclimated trout than in normal-calcium

acclimated trout. Injection of the residue proteins (crude tissue homogenate minus hypocalcin) do not affect Ca^{2+} influx.

CS crude tissue homogenates, hypocalcin, and residue proteins do not alter Ca^{2+}efflux compared to the saline treated or untreated fish (Figs 2 and 3). Saline infusion (control "e") do not alter Ca^{2+} efflux compared to untreated fish (control "c"; Figs 2 and 3).

Net Ca^{2+} flux from the water in low and normal calcium acclimated trout are indicated by hatched bars in Figs 2 and 3. Injection of crude CS tissue homogenates and of hypocalcin, but not of the residue result in a decrease of net Ca^{2+} flux. Actually, fish show a net loss of calcium after injection of crude tissue homogenate as a result of the decreased Ca^{2+}influx. (2.0 and 1.55 µmol per hour per kg fish for normal and low-calcium acclimated fish, respectively). The same doses of hypocalcin resulted in significantly greater inhibition of the Ca^{2+} net flux in the low-calcium acclimated fish (97 %) than in the normal-calcium acclimated fish (52 %).

Branchial Ca^{2+} influx.

Hormone effects. As shown in Table 1, crude tissue homogenates as well as isolated hormone, but not the residue proteins result in a statistically significant decrease of branchial Ca^{2+} influx in the isolated head preparation. For each fish the post-hormone treatment flux is expressed as a percentage of the pre-hormone treatment flux (=100 %), because of the considerable variability in the values for Ca^{2+} flux between individual fish. The average pre-treatment value was 7.28 ± 1.13 µmol per hour per kg fish (n=19; range 3.24 - 8.40 µmol/h/kg).

Hormone and control injections did not affect the TEP. For the control, crude tissue homogenate, hypocalcin and residue treated fish the TEP was -0.05 ± 1.19, -1.62 ± 0.76, -0.65 ± 0.77 and -0.09 ± 0.39 mV, respectively. The average value for all fish was -0.58 ± 0.70 mV.

Treatment with crude tissue extracts resulted in an increase of systemic blood pressure of 20.3 ± 6.3 %. No blood pressure effects were observed in control-, hypocalcin- or residue-treated isolated head preparations (non detectable, 4.6 ± 2.8 and -1.0 ± 3.3 %, respectively).

The following conclusions are drawn from the data presented in this paper:

- In freshwater rainbow trout, hypocalcin, the 54 kDa glycoprotein isolated from the CS, inhibits Ca^{2+} uptake from the water by inhibition of Ca^{2+} influx; Ca^{2+} efflux is not affected.
- Hypocalcin is more effective in fish acclimated to low-calcium water than in fish from normal-calcium water.
- Hypocalcin also inhibits Ca^{2+} influx in the isolated head preparation, thereby establishing the gill as an important target for hypocalcin.
- Hypocalcin does not alter the branchial TEP; we suggest, therefore, that the inhibition of Ca^{2+} influx is due to a specific modulation of the Ca^{2+} uptake mechanism in the gill.

Our values for whole body Ca^{2+} flux and branchial Ca^{2+} flux determined with the isolated head technique are in agreement with Ca^{2+} flux values reported in the literature (3, 5, 8, 13, 14, 15). We showed that hypocalcin, the 54 kDa product isolated from trout CS (Ch.3, 9) inhibits whole body Ca^{2+} uptake. Thus, the reduction of plasma calcium levels caused by injections of crude CS tissue homogenates observed in freshwater fish (21), at least in part, is the result of the hypocalcin induced reduction of whole body Ca^{2+} uptake.

The inhibition of calcium influx by hypocalcin in adult trout was a consistent phenomenon in our study. Wagner *et al.* (20) reported that the hypocalcemic principle of salmon CS exerts inhibitory effects on ^{45}Ca influx in juvenile rainbow trout, but only when basal Ca^{2+} influx in these fish is high (19). Our comparison of whole body Ca^{2+} flux in fish from normal-calcium and low-calcium water, with high and low Ca^{2+} influx rates, did not show such a relationship between Ca^{2+} influx and the inhibitory effect of hypocalcin.

We calculated that on protein basis four times more crude tissue extract than purified hormone was needed to obtain the same degree of inhibition of whole body Ca^{2+} influx. This indicates that hypocalcin was purified from crude tissue extracts by a factor of about 4. This seems a rather low purification factor. It is, however, in good

agreement with the relatively large amount of hypocalcin present in crude tissue homogenates of CS of trout and eel when analysed by SDS-polyacrylamide gel electrophoresis (about 12 % on protein basis, Ch.3, 9), and with the abundance of secretory granules observed in electronmicrographs of freshwater trout CS (Ch.3, 9, 22).

In trout injections of hypocalcin inhibited branchial calcium influx but did not induce significant hypocalcemia within the 4 hour period of the flux determination. This may be expected since the net whole body Ca^{2+} flux from water to fish is still positive. After injection of crude tissue extracts a net loss of calcium is observed. Assuming an extracellular fluid volume of 300 ml/ kg body weight (8), a loss of 8 µmol calcium as observed in a four hour period is only 1.3 % of the total calcium present in the extracellular fluid compartment of the fish and, therefore, may not be detectable at the level of a change in plasma calcium. The same dose of trout hypocalcin (6.5 fish equivalents), however, does evoke a hypocalcemic response in 15 days stanniectomised eels (Lafeber, unpublished results).

In trout adapted to low-calcium water, the effect of hypocalcin was more effective to inhibit Ca^{2+} influx than in fish from normal-calcium water. This is consistent with observations of Wendelaar Bonga *et al.* (21), on killifish and tilapia. These authors showed that CS homogenates hardly influence plasma calcium levels in fish from normal-calcium freshwater. Conversely, significant hypocalcemia could be induced in fish from low-calcium freshwater. It was suggested that in low-calcium water the circulating levels of the active hypocalcemic principle of the CS will be low, which enables exogenous hormone to be more effective (21). This interpretation may also be applicable to our results.

We showed that both Ca^{2+} influx and Ca^{2+} efflux were lower in fish from low-calcium water than in fish from normal-calcium water, whereas total calcium uptake was similar in both groups. In this respect trout differ from tilapia, in which increased Ca^{2+} influx and efflux were reported, resulting in an increased Ca^{2+} uptake, in low-calcium water (7). Apparently the control of calcium homeostasis is different in the two species.

The reduction of whole body Ca^{2+} uptake by hypocalcin is caused by a specific inhibition of branchial Ca^{2+} influx; whole body Ca^{2+} efflux is not effected. These results imply that the effect of hypocalcin on calcium extrusion *via* the kidney (1) must quantitatively be of less importance. It has been reported that calcium uptake by the gut is inhibited by hypocalcin (23). However, Ca^{2+} influx takes place for the larger part at the gill (4, 5) indicating the gill as an important target for hypocalcin. Therefore the relative contribution of the control of hypocalcin on the intestinal Ca^{2+} uptake will be of less importance for the whole body Ca^{2+} uptake.

The inhibition of hypocalcin on gill calcium influx, as we observed in the isolated head, can account for the effect observed *in vivo*. In the isolated head 21 % inhibition was obtained with 50 µg/100 ml Ringer. In the intact fish 52 % inhibition was obtained with 50 µg/100 g body weight. Assuming that the hypocalcin distribution space in the fish is equivalent to the extracellular fluid volume (300 ml/kg body weight; 8) the *in vivo* dose of 50 µg/100 g body weight corresponds to a dose of 150 µg/100 ml extracellular fluid. These calcultions indicate that the *in vivo* to *in vitro* dosage ratio (0.42) is comparable to the *in vivo* to *in vitro* percentage inhibition ratio (0.35). Thus the inhibition of gill calcium uptake can be explained by the inhibition of branchial calcium uptake. This further substantiates indicates that the gill is an important target for hypocalcin.

Since no pressor effects were observed with hypocalcin or residue proteins, we conclude that the increase in branchial vascular resistance is an effect specific for CS crude tissue extracts. The substance involved in pressure control must be smaller than 5 kDa (such small proteins are lost during ultrafiltration; Ch.3, 9) or inactivated during the isolation procedure, since no effect is observed after treatment with residue proteins. Chester Jones and coworkers had suggested already in 1969 the involvement of CS in vasopressor control (1). Apparently, hypocalcemic as well as vasoactive substances are present in the CS.

Our data show that hypocalcin is involved in the inhibition of branchial uptake. But how does hypocalcin affect branchial calcium flux? A model for branchial Ca^{2+} uptake in freshwater trout has been

postulated by Perry and Flik (15). This model essentially corroborates the model originally postulated for branchial Ca^{2+} uptake in tilapia (5, 6). In this model Ca^{2+} uptake is thought to include a passive apical Ca^{2+} influx *via* calcium channels, and an active basolateral Ca^{2+} extrusion from the cytosol in the blood *via* a high affinity Ca^{2+} ATPase. In our studies on the hormonal control of branchial Ca^{2+} uptake mechanism in freshwater fish, evidence is accruing, although no decisive answers can be given yet, that hypocalcin does not act on the basolateral Ca^{2+} ATPase. We therefore, hypothetize that the hypocalcin-induced inhibition of branchial Ca^{2+} influx involves modulation of calcium channels in the apical membranes of the calcium transporting cells.

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Chapter 9

EXPERIMENTAL HYPERCALCEMIA INDUCES HYPOCALCIN RELEASE AND INHIBITS BRANCHIAL Ca^{2+} INFLUX IN FRESHWATER TROUT

ABSTRACT

Intravascular $CaCl_2$ infusion in freshwater rainbow trout (*Salmo gairdneri*) causes a significant degranulation of the corpuscles of Stannius (CS). Concurrently, there is a specific and acute inhibition of whole body Ca^{2+} influx; Ca^{2+} efflux is unaffected. The material released consists primarily of a 28 kDa product which we identified as monomeric hypocalcin. Electron microscope observations of the CS reveal that type 1 and type 2 cells are degranulated to a similar extent. We conclude that hypocalcin is directly involved in hypocalcemic control in freshwater fish *via* inhibition of branchial Ca^{2+} influx, thereby promoting a net loss of Ca^{2+} across the gill.

Corpuscles of Stannius (CS) are small endocrine glands characteristic for holostean and teleostean fish. Rainbow trout usually have two to five corpuscles, located ventrocaudally to the kidney, that vary in diameter from 2 to 3 mm (1). More than the calcitonin secreting ultimobranchial glands, the CS are thought to be involved in hypocalcemic control in fish (see ref 2).

In the literature, a variety of secretory products has been reported to be secreted by the CS (see 3-5). However, it has been demonstrated that the hypocalcemic product released by the CS is a glycoprotein (6, Ch.3, 7). We have recently isolated the glycoprotein from trout (Ch.3, 7), and call the principle hypocalcin, the name originally proposed by Pang *et al.* (8). Trout hypocalcin shows only small differences in molecular weight and substantial similarity in the N-terminal amino acid sequence when compared with the hypocalcemic principle of salmon, isolated by Wagner *et al.* (6), and eel isolated by Butkus and colleagues (9).

There are indications that the CS of most euryhaline and freshwater fish species (such as trout), contain two structurally different cell types, *viz* type 1 and type 2 cells (3, 10). Type 1 cells have been reported to respond to changes in the calcium concentration of the ambient water and thus are presumed to be the cells secreting the hypocalcemic principle. There is no consensus on the role of the type 2 cells although the regulation of electrolytes other than calcium has been attributed to this cell type (11, 12). However, some authors have suggested that both cell types are simply different physiological stages of one functional cell type (*e.g.* 13).

Fish regulate their blood calcium levels efficiently (14, 15). They absorb calcium *via* the gills directly from the water and this uptake route accounts for most of their calcium requirement (16). Wagner *et al.* (6) showed that the salmon CS hypercalcemic principle decreases whole body ^{45}Ca uptake in juvenile rainbow trout. Intra-arterial injections of homologous hypocalcin inhibits gill Ca^{2+} influx in trout (Ch.8, 17). Although these data demonstrate the potential of hypocalcin to act as an hypocalcemic hormone, direct evidence for a

hypocalcemic function of endogenous hypocalcin is lacking.

In this paper we report that experimentally induced hypercalcemia in freshwater rainbow trout results in a rapid degranulation of the CS cells, which coincides with a rapid and specific inhibition of whole body Ca^{2+} influx. These data, together with the results of our previous study (Ch.8, 17) that showed inhibition of branchial Ca^{2+} influx by hypocalcin injections, strongly suggest that hypocalcin indeed fulfills a hypocalcemic role in the calcium homeostasis of freshwater fish.

MATERIALS AND METHODS

Animals.

Trout (*Salmo gairdneri*) used for whole body flux experiments (172 ± 5 g; n=35) were obtained from a commercial trout farm (Ontario, Canada). The fish were acclimated for at least 3 weeks to dechlorinated Ottawa city tap water containing (mM); Ca^{2+} (0.5), Cl^{-} (0.1), Na^{+} (0.1), K^{+} (0.02). Our set up allowed a constant circulation of water through 1000 liter fibre glass tanks. Water temperature varied between 10 and 12 °C and a pH between 7.0 and 7.4. The photoperiod was 12 hours of light alternating with 12 h of darkness. Fish were fed daily with a commercial trout diet. Fish were not fed 48 h before experimentation.

Cannulation. Fish were anaesthetised in sodium bicarbonate buffered (pH 7.4) ethylaminobenzoate (MS222; 0.1 g/liter). During the surgery, that lasted up to 10 min, fish were kept moist and the gills were irrigated with water containing MS222. Dorsal aortic cannulation was performed using standard techniques. The cannula was secured to the roof of the buccal cavity with to stiches and led out of the mouth through a perforation in the snout of the fish. When not used for blood sampling, the cannula was filled with slightly modified Cortland saline containing 10 U/ml ammonium heparin. Fish were allowed to recover for at least 24 hours prior to experimentation in individual opaque perspex flux boxes (volume = 3 l) supplied with flowing water.

Experimental protocol.

Calcium challenge. Trout were infused *via* a dorsal aorta cannula with 1 ml per 100 g fish of a 0.07 M $CaCl_2$ solution (t= -1 h). Infusion

of a NaCl solution of a similar osmolarity (0.12 M) served as control. In the $CaCl_2$ infused group, two blood samples (200 µl) were withdrawn before infusion and 10 blood samples (200 µl) were withdrawn at regular intervals over a 5 hours period after infusion. Blood samples were centrifuged and plasma was analysed for total calcium content.

NaCl and $CaCl_2$ infused fish were used for whole body Ca^{2+} influx and efflux determinations. Ca^{2+} flux determinations were started 1 hour after the calcium infusion (t= 0 h) and lasted for 4 hours, upon completion of the experiment the CS were removed. A part of the glands was homogenized in 0.05 M ammonium acetate (pH 7.4) and prepared for SDS-PAGE (see below). Anothers part was prepared for electron microscope examinations.

Determination of whole body Ca^{2+} fluxes

Experimental procedures. Whole body Ca^{2+} flux was determined on cannulated fish kept in the flux boxes. At the start of a Ca^{2+} influx experiment, water flow through the flux box was discontinued and ^{45}Ca was added to the water (5×10^5 Bq/l; 1 h after $CaCl_2$ injection, t= 0 h). Efficient mixing of the ^{45}Ca with the water was assured by vigorous aeration of the water in the flux boxes. After a 4 hour exposure period the fish was killed with an overdose of MS222 (0.8 g/l; t= 4 h). Next the fish was rinsed in tap water (1 min) and transferred for 3 min to water containing 10 mM $CaCl_2$ to remove ^{45}Ca adsorbed to integumental surfaces. To determine total body ^{45}Ca activity, fish were microwave cooked (1.5 min) and homogenized in a commercial blender with 65% body weight of distilled water. Quintuple homogenate samples (approximately 0.5 g weighed to the nearest 3 decimals) were dissolved at 60 °C overnight in 2 ml of tissue solubiliser (NCS, Amersham). Subsequently the samples were neutralized with glacial acetic acid; 5 ml of distilled water was added and the ^{45}Ca content determined by liquid scintillation analysis (LSA).

For Ca^{2+} efflux experiments, fish were infused (*via* their cannula) 24 h before the start of the flux period with 2.22×10^6 Bq of ^{45}Ca in 1 ml of 0.6% NaCl. Ten min before the start of an experiment water flow through the boxes was discontinued and ^{45}Ca appearance rate in the water monitored (see below). After a flux period of 4 hours, the ^{45}Ca containing water was replaced by a flow of tracer free water until the

level of ^{45}Ca had returned to background levels. This first flux measurement served to determine control Ca^{2+} efflux. Subsequently, fish were infused with a $CaCl_2$ solution or NaCl solution as described above, and this was followed after 1 hour by a second 4 h flux period; the second flux period was used to determine the effects of NaCl or $CaCl_2$ infusions on Ca^{2+} efflux.

Blood and water sampling. For influx as well as efflux measurements blood samples of 500 µl were taken *via* the catheter immediately before the start of the flux period (t= 0 h) and upon completion of the experiment (t= 4 h). For influx determinations, 5 ml water samples were taken 15 min after the start of the flux period, and at the end of the flux period (t= 4h). For efflux determinations water samples were taken at the start of the flux period, mid-way and at the end of the flux period (t=0, 2 and 4 h, respectively). Blood samples were centrifuged in heparinized tubes (1,5 ml; Eppendorf). Triplicate 100 µl plasma samples and the 5 ml water samples were analysed for ^{45}Ca specific activity (SA). Influx of Ca^{2+} was calculated on the basis of the total tracer content of the fish after 4 h exposure to ^{45}Ca containing water and the mean specific activity of the water, and was normalized to fish weight. Ca^{2+} efflux was calculated from the slope of the time curves of tracer appearance in the water and the mean ^{45}Ca SA of the blood plasma, normalized to fish weight, and expressed in µmoles per hour per kg fish.

Analytical methods.

The total calcium content of water and plasma was determined with a commercial calcium-kit (Sigma). Combined calcium-phosphate standard (Sigma) was used as a reference.

^{45}Ca activity of water (5 ml) and plasma samples (100 µl plasma plus 4 ml of distilled water) was determined by LSA, after addition of 10 ml of Scinti-Verse II (Fisher).

Sodiumdodecylsulphate-polyacrylamide gel electrophorese (SDS-PAGE) was performed using polyacrylamide slab-gels (15%) run under reducing conditions, according to Laemmli (18). After fixation of the proteins, the gels were silver-stained as described by Morrissey (19), quantification was performed by densitometric scanning.

CS were prepared for electron microscopy according to Wendelaar Bonga *et al.*, (20). To discriminate between type 1 and type 2 cells ultrastructural criteria were used as described by Meats *et al.* (1) and Wendelaar Bonga *et al.* (20). Sections of CS of 5 different control (NaCl-infused) and experimental ($CaCl_2$-infused) fish were examined. Fifteen individual type 1 and type 2 cells were analysed per fish. Granule area as a fraction of cytoplasmic area was scored as a measure for granulation.

Statistical evaluation.

Data are presented as mean values ± SEM. For statistical evaluation the Mann-Whitney *U*-test was used. Significance was accepted at $P < 0.05$.

RESULTS

Shown in figure 1 the effects of $CaCl_2$ infusions on trout plasma calcium levels. Ten min after infusion a plasma calcium concentration of about 5 mM was reached. However, one hour after infusion plasma calcium levels had returned to 2.22 ± 0.07 mM, which is slightly, but significantly, above control values (1.73 ± 0.06 mM), and did not change significantly over the next 4 hour period (average value 2.31 ± 0.05 mM), in which flux determinations were performed.

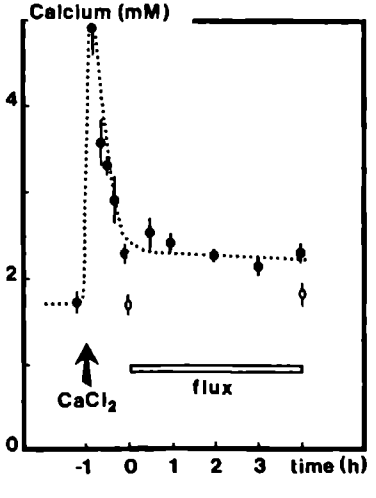
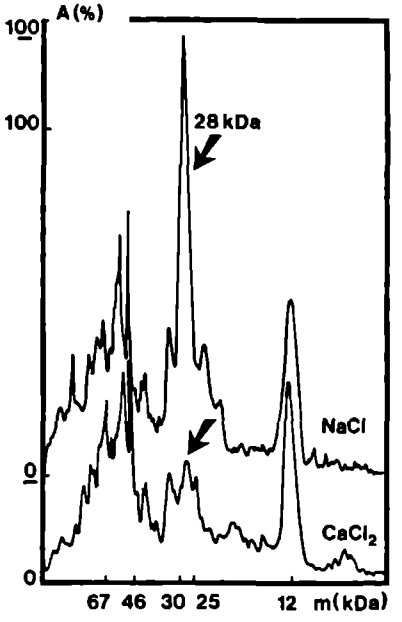


Fig 1. Plasma calcium concentrations in trout after $CaCl_2$ infusion (o), NaCl infusion (o). Mean values ± SEM are given; n=6. Bar indicates period of flux determinations.

Densitometric scans of the silver-stained proteins present in CS tissue homogenates of fish infused with either NaCl (a) or $CaCl_2$ (b) is given in figure 2. CS of trout infused with calcium are specifically devoid of a protein with an apparent molecular weight of approximately 28 kDa (gels run under reducing conditions).



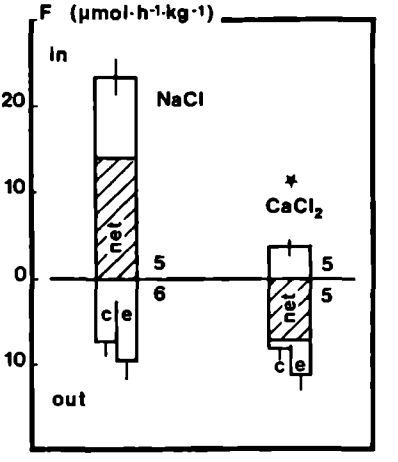


Fig 2. Densitometric scan of silverstained CS tissue homogenates of NaCl- and $CaCl_2$-infused fish after SDS-PAGE. Absorption is shown in % of maximum peak hight (underlined percentages refer to NaCl injection). Molecular weight (*m*) of protein markers are given on the horizontal axis. Arrow indicates 28 kDa hypocalcin.

Fig 3. Calcium influx and calcium efflux for NaCl and $CaCl_2$ infused fish; c = efflux before infusion, e = efflux after infusion. Hatched bars indicate net flux. Mean values ± SEM are given; numbers indicate n. Asterisk indicates statistically significant difference from NaCl-infused fish.

Figure 3 shows the effects of a calcium challenge on unidirectional whole body Ca^{2+} fluxes. A specific inhibition of calcium influx was observed in the $CaCl_2$-infused fish; Ca^{2+} efflux was not effected by calcium treatment.

Both, type 1 and type 2 cells of the CS had degranulated after $CaCl_2$ infusion (figure 1). Type 1 cells show a 52 % degranulation as compared to the controls; whereas type 2 cells show a 41 % degranulation.

Table 1. Effects of $CaCl_2$ infusions and NaCl infusions on the degree of granulation of the CS.

treatment	granulation (%)	
	type 1 cells	type 2 cells
NaCl	15.40 ± 1.14	2.02 ± 0.17
$CaCl_2$	7.38 ± 0.72 *	1.20 ± 0.33 *

Mean values ± SEM are given (n = 5). Asterisks indicate statistically significant different from NaCl infused controls. Granulation (at t = 4 h) is expressed as the percentage granule area of the cytoplasmic area.

DICSUSSION

Experimentally induced hypercalcemia in freshwater trout results in the release of hypocalcin by the CS, and a subsequent inhibition of gill Ca^{2+} influx, whereas Ca^{2+} efflux is not effected. This results in a net loss of calcium from the fish. Plasma calcium levels are restored rapidly after $CaCl_2$ injections: within one hour after injection plasma calcium levels are only slightly above control levels. It is unlikely, however, that this rapid decrease of plasma calcium is caused by the observed inhibition of Ca^{2+} influx. One may calculate from the data in fig 3 that the magnitude of net Ca^{2+} loss (approximately 8 µmol/h/kg), and that this rate is too low to explain the plasma calcium decrease of 1 mmol/h/kg observed in the first hour after $CaCl_2$ injection. The lattervalue is calculated from fig 1 assuming an extracellular fluid volume of 300 ml/kg fish; 21. It is likely, therefore, that the rapid

decrease in plasma calcium within the first hour after $CaCl_2$ injection is mainly due to equilibration with rapidly exchanging internal calcium pools, such as scales, bone and soft tissue (22).

Measurements of Ca^{2+} fluxes in our control trout are in good agreement with recent studies on this species (Ch.8, 17, 23, 24). The reduction of whole body Ca^{2+} influx following $CaCl_2$ injection is a rapid response. This is in line with our assumption that this inhibition is mediated by hypocalcin, releaased upon the calcium challenge: the reduction of Ca^{2+} influx following injections of exogeneous hypocalcin is also an immediate effect that becomes noticable within a similar time span (Ch.8, 17).

In the present study it was observed that degranulation of the CS results in the release of a product with an apparent molecular weight of 28 kDa. We have indentified this product earlier as the reduced form of hypocalcin (Ch.3, 7). The secretion of this product seems specifically related to the increased plasma calcium concentration; magnesium or phosphate infusions do not induce the release of hypocalcin (unpublished observations). Whether this effect of increased plasma calcium levels on the release of hypocalcin is direct or indirect remains to be established.

The concomitant degranulation of the CS cells and the specific inhibition of Ca^{2+} influx after experimentally induced hypercalcemia leads us to conclude that hypocalcin modulates branchial Ca^{2+} influx in the trout. The mechanisms involved in the hypocalcin-mediated inhibition of Ca^{2+} influx remain ill-defined. However, the rapidity of the effect excludes changes in the modulation of the amount of branchial Ca^{2+}-ATPase, and recent data (G. Flik, personal communication) fail to show effects of hypocalcin on the kinetic properties of the Ca^{2+}-ATPase. As suggested by Perry and Flik (24) and Lafeber *et al.* (Ch.8, 17), the inhibition of Ca^{2+} influx by hypocalcin most likely involves a decrease in apical membrane permeability in the calcium transporting cells of the gills, presumably by affecting apical membrane calcium channels (24).

We feel that it is unlikely that heamodynamic adjustments contribute to the reduced influx because Ca^{2+} efflux is unaffected and hypocalcin does not affect perfusion pressure in an isolated trout head preparation (Ch.8, 17).

We show here that type 1 cells as well as type 2 cells of the corpuscles degranulate after $CaCl_2$ injection. Also Lopez and coworkers (13) concluded that both secretory cell types present in eel CS are degranulate after a six- to seven-fold increase of plasma calcium, induced by $CaCl_2$ injection. These authors concluded on the basis of light microscope observations that both types represent structurally different stages of one functional cell type. The size and number of the granules of the type 2 cells are such that these cells usually appear as non-stainable cells in the light microscope, and therefore ultrastructural examination seems a prerequisite for the study of degranulation of these cells. However, on the basis of our morphometric analysis we also conclude that the degree of degranulation is similar for both cell types. Several explanations are possible for this observation.

First, it could be argued that the response of the CS cells (both type 1 and type 2 cells) to hypercalcemia is non-specific. It is well known that in many types of secretory cells granule release can be induced, at least *in vitro*, by a moderate increase of the calcium concentration of the ambient medium (25). Extremely high calcium levels, as induced in our experimental animals, usually lead to inhibition of glandular secretion (26, 27). In our experiments preliminary results showed no differences between the pituitary prolactin cells and the ultimobranchial C-cells of control and $CaCl_2$ injected trout. In contrast, a marked degranulation was found in both types of CS cells. This response indicates a specific and direct relationship between the rates of secretion of both cell types and plasma calcium.

An alternative explanation is that both cell types produce different hormones but share the specific response to hypercalcemia. To date, only one hormonal substance has been isolated from the CS (6, Ch.3, 7). The study of the biosynthesis of the CS *in vitro* revealed synthesis of only one major product with a molecular weight of 28 kDa (28) which has subsequently been shown to represent hypocalcin of the CS (Ch.3, 7). However, the small size and number of the granules of the type type 2 cells, when compared with the numerous large granules of the type 1 cells, reflect a low production of the secretory product of the type 2 cells. Therefore, it is possible that this product may not

be detected by biochemical analysis, and the possibility that both cell types produce different substances cannot be excluded.

As a third explanation it could be debated that both cell types represent two structurally different subtypes or phases of the same functional cell type, which has been suggested before. On the basis of electron microscope studies the presence of two cell types, different in size and in the amount of and distribution of the secretory granules, was shown for the gold fish (29), guppy (30), Atlantic salmon (31), toadfish (32), and Coho salmon (33). It was suggested by these authors that the two cell types represent different structural stages of one functional cell type. However, physiological support for this suggestion has hardly been provided in any of these studies. On the other hand, several authors have reported that both cell types respond differently to changes in electrolyte concentrations of the ambient water (1, 11, 20, 34, 35), and it was proposed that they produce different hormones. Our observations represent the first ultrastructural evidence that both type 1 and type 2 cells respond to changes in plasma calcium. This urges a reinvestigation of the hypothesis that type 1 and type 2 cells are functionally similar.

It could be that hypocalcemic substances other than those of the CS are secreted during experimentally induced hypercalcemia. In this respect, calcitonin should be mentioned. We feel that it is unlikely that increased release of calcitonin is responsible for the rapid decrease of Ca^{2+} influx after injections of $CaCl_2$. In general only minor effects (if any at all) of calcitonin on blood calcium levels in fish have been reported (see 2, 3). Moreover, recently Fenwick and co-workers were unable to show effects of calcitonin on Ca^{2+} fluxes in the freshwater eel (personal communication). Contradictory results have been reported by Milet *et al.* (36). They showed an inhibitory effect of calcitonin on Ca^{2+} influx in isolated gill arches of the eel. However, in these experiments an even greater stimulatory effect on Ca^{2+} efflux was observed, an effect which we did not observe in our experiments. This makes it unlikely that the inhibition of $Ca2^{+}$ influx we observed after $CaCl_2$ injections was mediated by calcitonin.

In contrast to calcitonin, administration of CS extracts has consistently been reported to decrease plasma calcium levels (*e.g.* 3, 12, Ch.8, 17). Furthermore, we recently observed a rapid decrease of

Ca^{2+} uptake after intravascular infusions of hypocalcin; Ca^{2+} influx was inhibited by about 80 %, whereas Ca^{2+} efflux was not effected (Ch.8, 17). The results are very similar to the effects of $CaCl_2$ infusion on the Ca^{2+} fluxes observed in this paper. Therefore, it seems likely that the decrease in Ca^{2+} influx observed after an experimentally induced hypercalcemia results from the release of hypocalcin from the CS cells.

In the first paragraph of the discussion we concluded that the inhibition of Ca^{2+} influx that occurred after $CaCl_2$ injections resulted in a net efflux of Ca^{2+} of approximately 8 µmol/h/kg. This is equivalent to a decrease of the total plasma calcium concentration with approximately 27 µM per hour (assuming an extracellular fluid volume of 300 ml/kg fish). We ascribe this effect to the release of hypocalcin from the CS, and conclude than that the capacity of the CS to release hypocalcin seems sufficient for effective control of plasma calcium levels under physiological conditions.

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The relationship between the corpuscles of Stannius and the parathyroid glands

PTH-like activity of hypocalcin

The results presented in the Ch.2 and Ch.4 show that there is a striking similarity between the bone resorbing activity of hypocalcin and PTH in mouse calvaria. CS homogenates and hypocalcin evoked similar responses whereas residue proteins did not, from which we concluded that hypocalcin is responsible for the PTH-like activity of the CS extracts.

Hypocalcin was able to increase bone resorption by the enhancement of osteoclastic activity. Approximately 430 times more hypocalcin than PTH was required to obtain a similar bone resorbing effect (Ch.4). Proceeding from this potency, the dose response curves for both hypocalcin and PTH were similar. Furthermore, no additive effect was observed when maximum stimulating concentrations hypocalcin and PTH were tested together. It is therefore likely, although no decisive data can be shown, that both hypocalcin and PTH act *via* the same receptor.

This conclusion seems to be in contrast with the observation that hypocalcin is unable to stimulate the production of cAMP (Ch.4), because cAMP reportedly is an important second messenger in the PTH induced bone resorption (1). However, it has recently been found that the relation between PTH and cAMP is complicated. PTH-induced bone resorption involves activation of osteoclasts by intervention of osteoblasts (2, 3). The mode of signalling from osteoblast to osteoclast remains to be elucidated. Activation of the osteoblast involves increase of intracellular cAMP and Ca^{2+} levels in these cells (4-6). The actions of both second messengers are interrelated, although either of them is able to induce bone resorption *in vitro* (7). Evidence has been obtained for the hypothesis that PTH induced bone resorption involves activation of an osteoblast receptor with two binding sites or two distinct receptors (Fig 1; 6-8). One of these receptor sites is

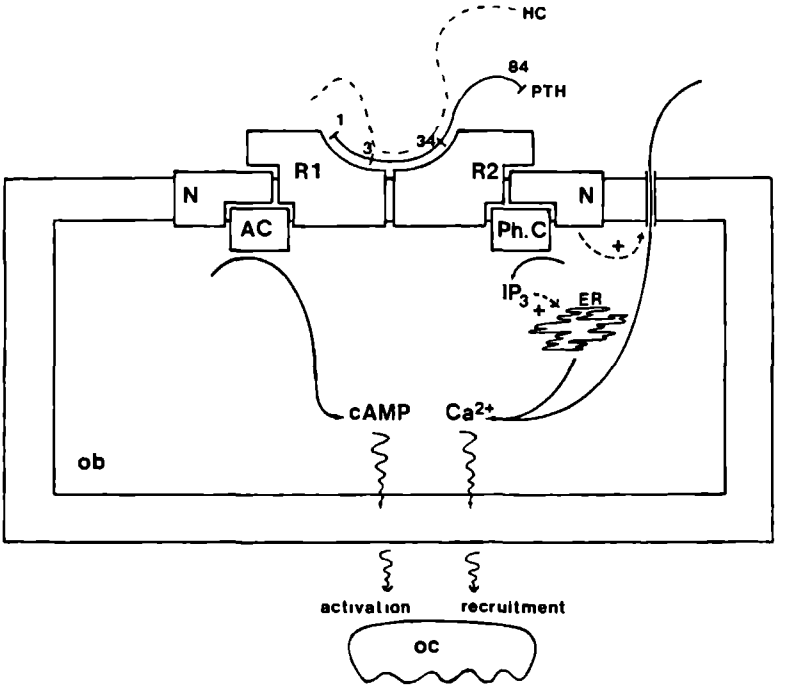

Fig 1. R1, receptor site which can be activated by PTH when the first two N-terminal amino acids are present; R2, receptor site activated by PTH when amino acid 3-34 are present and activated by hypocalcin (HC); N, guanine nucleotide regulatory unit; AC, adenylate cyclase; Ph.C, phospholipase-C; IP_3, inositol-triphosphate; ER, endoplasmic reticulum; ob, osteoblast; oc, osteoclast. Ativation of both receptors results in a full physiological response in osteoblasts. At first cAMP production results in activation of the existing osteoclasts; subsequently, Ca^{2+} activity results in recruitment of osteoclasts. Ca^{2+} may be released from the endoplasmic reticulum by IP_3 and/or may enter the cell *via* calcium channels in the outer membrane. *(modified after Löwick et al. 1985 and Herrmann-Erlee et al. 1988.)*

activated by the N-terminal part of the PTH molecule (including the first 3 amino acids) and mediates adenylate cyclase activation. The other receptor site is thought to be activated by the C-terminal part of PTH (including aminoacids 4-34) which leads to the activation of the Ca^{2+} second messenger pathway. The phospho-inositol metabolism (induction of inositol triphosphate by activation of phospholipase-C) could be involved in activation of the Ca^{2+} second messenger pathway (9). Involvement of guanine nucleotide regulatory proteins in this receptor second messenger system is suggested (10).

Surprisingly, PTH shortened or modified at the N-terminal part, *e.g.* PTH(3-34), does not effect cAMP production, but is still able to induce bone resorption. This fragment was shown to act *via* Ca^{2+} as a second messenger (6, 8). Since hypocalcin neither stimulates cAMP production, it may also induce bone resorption *via* Ca^{2+} as a second messenger. This hypothesis could indirectly be confirmed; hypocalcin increases the number of osteoclasts (Ch.2, 4) which is suggested to be regulated through Ca^{2+} functioning as a second messenger (8). These data suggest that hypocalcin could act *via* the same receptor as (N-terminal shortened) PTH in bone resorption, although direct evidence is still lacking. The observation that hypocalcin stimulates bone resorption in a PTH-like fashion but does not stimulate cAMP production makes hypocalcin an appropriate and interesting tool to investigate the bone resorbing mechanism and the relative contribution of both receptor sites.

Hypocalcin-like activity of PTH

Both hypocalcin and bovine PTH(1-84) show blood calcium lowering activity in STX eels (Ch.5). Equimolar concentrations of hypocalcin and PTH gave similar effects. This result corroborates previous results obtained in our laboratory which showed that bovine PTH(1-34) decreases plasma calcium levels in low calcium acclimated killifish and tilapia (11).

Hypocalcin induces its hypocalcemic effect mainly by the inhibition of calcium uptake *via* the gills from the water (Ch.8). In contrast, bovine PTH(1-34) was unable to inhibit whole body Ca^{2+} uptake from the water (unpublished results). PTH(1-84) has not been tested yet. The absence of an inhibitory effect of PTH(1-34) might be explained by the supposition that the C-terminal part of PTH is essential to mimic the inhibitory effect of hypocalcin on whole body calcium uptake, whereas the N-terminal part evokes a hypocalcemic response different from the effect induced by hypocalcin. This hypothesis is supported by an observation reported in Ch.5: we showed that in hypocalcin treated fish the decrease in ionic calcium fully accounted for the decrease in total calcium, whereas in the PTH treated fish the decrease in total calcium exceeded the decrease in ionic calcium. This implies that PTH(1-84), in addition to the reduction of

blood ionic calcium, reduces the protein bound calcium fraction of the blood. In spite of this difference to hypocalcin, the similarity between the effects of hypocalcin and PTH in fish is remarkable.

Comparison of hypocalcin and PTH

There is not only a striking similarity in bioactivity between hypocalcin and PTH in fish: also the molar concentration of PTH that is required to obtain an effect in fish is comparable to that of hypocalcin (Ch.5). Moreover, although the potency of hypocalcin was low when compared to that of PTH in our bone resorption assay, the molar ratio of 430:1 that we found (Ch.4) compares well with *e.g.* the molar ratio of approximately 1000:1 reported for ovine prolactin in a fish prolactin bioassay (12, 13). What then is underlying this similarity in bioactivity?

The hypothesis that the CS and parathyroids are homologous, as postulated by Lopez and colleagues (see ref 15), is no longer tenable. The homology has been questioned before (14) because both glands reportedly had a different embryological origin, the renal mesoderm and the neural crest, respectively. However, the recent demonstration in the CS and the PTH glands of immunoreactivity to secretory protein I, a neural crest marker, indicates that the embryological origin of both glands may indeed be similar (15). Nevertheless, the observation that the corpuscles of Stannius are confined to holostean and teleostean fish seems to exclude that the CS are phylogenetically related to the parathyroids. The terrestrial vertebrates have evolved from ancestral forms of the Sarcopterygii and not from the Actinopterygii to which the Holostei and Teleostei belong (16).

Irrespective of the embryological origin of both glands, the fact that hypocalcin and PTH have no similarity in primary structure contradicts the suggested homology between both calcium regulating glands. As discussed in chapter 3 and 4, the amino acid sequence as predicted from DNA structure of eel hypocalcin, and the N-terminal amino acid sequence of trout hypocalcin presented in chapter 3 show striking similarity with each other but not with PTH. Homology of the two hormones can therefore be excluded.

It may be concluded that hypocalcin and PTH are a unique type of agonists. Hypocalcin mimics the effect of PTH and PTH mimics the effect

of hypocalcin. We therefore suggested that the similarity in bioactivity originates from a similarity in the three dimensional structure of both molecules (Ch.3). It is very interesting that two structurally unrelated peptides have similar bioactivity in fish as well as in mammalian test systems. It is known that unrelated molecules can evoke similar responses, *e.g.* morphines (alkaloids) mimic the effects of endogenous peptides, like endorphins and enkephalin, on the opiate receptors (17). It is, however, a unique and fascinating possibility that the ternary structure of two peptide hormones is responsible for similarity in bioactivity.

Mechanisms involved in hypocalcemic control

Hypocalcin and hypocalcemic regulation in freshwater fish

Removal of the CS from eels resulted in a prolonged hypercalcemia, which was not corrected within at least 5 weeks (Ch.5). We concluded from this observation that the CS produce the main hypocalcemic hormone in these fish (Ch.7). Furthermore, experimentally induced hypercalcemia caused the release of hypocalcin from the CS and concurrently inhibited whole body calcium uptake (Ch.9). This effect on calcium uptake was very similar to the effect observed on whole body calcium uptake of fish that were injected intra-arterially with hypocalcin (Ch.8), which indicates that hypocalcin release under normal conditions will be evoked by elevated plasma calcium levels.

Our data showed that the inhibition of whole body calcium uptake by hypocalcin was mainly caused by a decrease in gill calcium uptake (Ch.8). The calculated net loss of calcium caused by hypocalcin was sufficient to draw the conclusion that hypocalcin is a potent hypocalcemic hormone, with the gill as the most important target organ. This implies that hypocalcemic regulation in fish is mainly effected by modulation of the branchial exchange of calcium between the fish and the ambient water. It does not fully exclude an involvement of gut, kidney and bone in hypocalcemic regulation, but the contribution of these organs must be of minor importance in fresh water fish. On the other hand, the specific location of the CS on or in the kidneys and

the observation that the blood comming from the CS is drained *via* the kidneys (14) suggests that the kidneys are a target organ for some CS factor other than hypocalcin. It could be that the pressor activity that we found in a crude CS tissue homogenate and which could not be ascribed to hypocalcin (Ch.8), is exerted by an unknown substance produced by the CS that is involved in renal function, *e.g.* regulation of the glomular filtration rate (18).

The mechanism of action of hypocalcin is distinctly different from that of calcitonin, the main hypocalcemic hormone of the terrestrial vertebrates. Although calcitonin is present in fish, and salmon calcitonin is used for anti-hypercalcemic treatment in clinical medicine, in fish calcitonin stimulates bone formation but is not directly involved in blood calcium regulation (19, 20). The function of calcitonin as a major hypocalcemic hormone probably developed after the water-to-land transition in vertebrate evolution, when the water was no longer available as a readily accessible calcium source and bone became important for calcium homeostasis of the body fluids. The function of calcitonin in calcium homeostasis may have evolved in conjunction with that of PTH, the homeostatic antagonist of hypocalcin in the terrestrial vertebrates.

Hypocalcin inhibits branchial Ca^{2+} uptake

Hypocalcin is involved in short term inhibition of branchial Ca^{2+} uptake. But how does hypocalcin effect branchial calcium uptake? A model for branchial Ca^{2+} uptake, as originally postulated for tilapia and subsequently modified for trout (Fig 2.), proposes a passive Ca^{2+} entry along an electrochemical gradient from the water across the apical membrane of the chloride cells into the cytosol (21, 22). This diffusive Ca^{2+} influx, which is facilitated by the high Ca^{2+} gradient over the apical membrane, was shown to occur *via* voltage-independent calcium channels (22). Calcium transition from the cytosol to the blood over the basolateral membrane of the chloride cells (an active process) is reported to be achieved through a Ca^{2+} ATPase pump mechanism. Branchial Ca^{2+} efflux on the other hand is thought to be a paracellular process controlled by the trans-epithelial potential (TEP) and the chemical gradient for Ca^{2+} between the extracellular fluid and the ambient water.

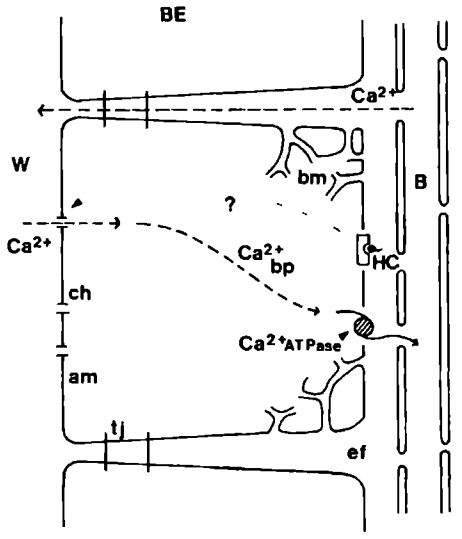

Fig 2. Model for branchial calcium uptake. BE, branchial epithelium; W, ambient water; B, blood; ef, extracellular fluid; am, apical membrane; bm, basolateral membrane; ch, Ca^{2+} channel; tj, tight junction; HC, hypocalcin; Ca^{2+}bp, calcium binding proteins. Hypocalcin activity (receptor activation and second messenger activity) results in blockage of apical calcium channels and a subsequent inhibition of branchial Ca^{2+} influx. *(Model modified after Flik et al. 1985 and Perry and Flik 1988).*

We hypothesize that hypocalcin acts by inhibition of the calcium entry into the chloride cells *via* an effect on the permeability of the apical membrane. The following arguments support this hypothesis. In our calcium flux studies no effects were observed on Ca^{2+} efflux, from which we conclude that hypocalcin does not effect the passive loss of Ca^{2+} (Ch.8, 9). This was supported by the fact that administration of hypocalcin did not influence the trans-epithelial potential across the branchial epithelium (Ch.9). Branchial influx of Ca^{2+}, however, was inhibited rapidly. On the basis of the model presented in figure 2, the effect of hypocalcin on Ca^{2+} influx can be due to either a direct inhibition of the Ca^{2+} transport ATPase in the basolateral membrane or an inhibition of the calcium entry at the apical membrane of the calcium transporting cells. The hypocalcin-induced inhibition of branchial Ca^{2+} influx might involve modulation of either the calcium channels at the apical membrane or the calcium pumps in the basolateral membranes, or both. The first possibility is the most likely one. Changes in the basolateral transport capacity by a change in the amount

of transport Ca^{2+} ATPase, *e.g.* by decreasing the number of cells or the amount of ATPase per cell, is unlikely within the time span of the experiment. Moreover, recently it has been found in our laboratory that a direct inhibition of the basolateral Ca^{2+} transport activity could not be established, whereas hypocalcin instantaneously inhibited the influx of Ca^{2+} in the branchial epithelial cells (Verbost, preliminary unpublished results).

A model for endocrine calcium regulation

The results presented in this thesis show that fresh water teleost fish possess a fast acting hypocalcemic control mechanism, which enables them, *via* the release of hypocalcin, to decrease quickly the uptake of calcium from the water. As shown in Ch.9, the final result of the release of hypocalcin may be a net loss of calcium. It should be kept in mind, however, that fresh water fish live in an environment with a calcium concentration slightly or well below that of the blood plasma. Since there is a passive loss of calcium (Ch.8) caused by outward diffusion across the gill epithelium one could argue that freshwater fish are facing a calcium shortage, unless the losses are compensated for by active calcium uptake. From this point of view the significance of hypocalcemic control mechanisms, and thus the functional importance of the CS, seems questionable. For growth and reproduction, and for compensation of the passive calcium losses a constant uptake of calcium from the environment is necessary. This seems to require hormones that promote calcium uptake, and any hormone inhibiting this process seems superfluous. What then is the function of the CS and of hypocalcin in freshwater fish?

The supposition that freshwater fish are facing a calcium uptake shortage may be wrong. Although the calcium concentration of the blood plasma is higher than that of the water, the calcium concentration in the cytoplasm of the chloride cells separating the water from the blood is lower by at least two orders of magnitude (assuming that the micromolar concentration present in the chloride cells is similar to the concentrations required for proper cell function in general). The model for branchial Ca^{2+} uptake as proposed above implies that calcium can enter the chloride cells passively. These cells are therefore at

risk of being incapacitated by excess calcium, unless the apical entry of calcium is balanced by the basolaterally located Ca^{2+} translocating mechanism. This leads to the conclusion that the control of the calcium uptake capacity across the branchial epithelium requires control of both the passive entry of calcium over the apical membrane as well as the active translocation of calcium across the basolateral membrane.

We concluded above that hypocalcin controls the passive entry of calcium across the apical membrane. Prolactin is at least one of the hormones regulating the capacity of basolateral calcium translocation (23). However, this hormone seems less suitable for rapid modulation of the blood calcium level, since the effects of prolactin develop only slowly (24). The presence of a rapidly acting hypercalcemic hormone, possibly released from the pituitary gland, has been postulated, but not demonstrated so far (25, 26).

We suggest that a rapidly acting hypercalcemic hormone is not necessary for the endocrine control of plasma calcium levels. The presence of a hypocalcemic hormone, hypocalcin, seems sufficient to maintain plasma calcium levels. This hypothesis is based on the assumption that calcium will enter the fish *via* the gills and thus create hypercalcemia unless the entry is inhibited by hypocalcin. As stated above, the calcium can enter the chloride cells of the gills passively, *via* the apical membrane, and will be subsequently transported from the cell to the blood *via* the basolateral calcium pump. This ATPase dependent transport, however, is calmodulin dependent as has recently been demonstrated in our laboratory (26). This implies that the calcium pump can be stimulated directly, without hormonal interference, by a rise in cytoplasmic calcium. Thus, increased permeability of the apical cell membrane for Ca^{2+}, through a reduction in circulating hypocalcin levels, will result in hypercalcemia. A specific and rapid hypercalcemic hormone is not necessary to explain the relatively high plasma calcium levels in fresh water fish. We hypothesise, therefore, that calcemic control is effected by the hypocalcemic mechanism alone.

There is important evidence that supports this hypothesis, namely the excessive hypercalcemia that occurs after removal of the Stannius corpuscles. This phenomenon, for a long time not understood, is caused by an increase of the calcium uptake from the water (27). Apparently if

the passive calcium entry in the chloride cells is no longer restrained by hypocalcin, there is a lack of direct control of the calcium uptake from the water. This can be explained by assuming an overstimulation of the basolateral calcium transport mechanisms in the chloride cells due to chronically high intracellular calcium levels. Such a mechanism would lead to the rather paradoxical situation that a hypocalcemic hormone can be used for hypocalcemic as well as hypercalcemic control.

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I) Vergelijking van hypocalcine en PTH in zoogdier en vis bioassays.

Gewervelde landdieren zijn in staat het calciumgehalte van het bloed te reguleren binnen nauwe grenzen. Een tekort aan calcium in het bloed moet worden aangevuld omdat calcium voor veel processen van direct belang is *(hypercalcemische regulatie)*. Een teveel aan calcium heeft daarentegen schadelijke gevolgen en moet daarom worden weggewerkt *(hypocalcemische regulatie)*. Een goede afstemming van hypocalcemische en hypercalcemische regulatie leidt er toe dat het calciumgehalte in het bloed constant blijft *(calciumhomeostase)*.

Landdieren nemen calcium op via het voedsel en verliezen calcium met name via de urine. De opname van calcium via het voedsel verloopt niet continu. Een teveel aan opgenomen calcium moet worden opgeslagen om bij een tekort aan calcium in het bloed weer te worden afgegeven. Deze opslagplaats voor calcium wordt gevormd door het bot. Het hormoon *calcitonine* zorgt daarbij voor de opslag van calcium in het bot waardoor het calcium niveau in het bloed daalt. Daarentegen zorgt het bijschildklier hormoon *(parathyroid hormoon, PTH)* voor het vrijmaken van calcium uit het bot. Dit hormoon zorgt verder voor verhoging van het calcium gehalte in het bloed door de uitscheiding van calcium via de urine te verminderen en door de opname van calcium via de darm te stimuleren (indirect, via vitamine-D).

Ook vissen reguleren calcium gehaltes in het bloed. Vissen nemen calcium echter niet of nauwelijks op via het voedsel, maar direct uit het water, via de kieuwen. Zij verliezen calcium via de urine en via de kieuwen. De kieuwen zijn voor vissen dus zowel van belang voor de ademhaling als voor het opnemen van calcium. Regulatie van de opname van calcium door de kieuwen en van de afgifte van calcium via kieuwen en nieren kan dus zorgen voor een constant calciumgehalte in het bloed. Omdat deze regulatie processen bij vissen continu kunnen plaatsvinden is een opslagplaats voor calcium, zoals bij landdieren, niet noodzakelijk. Bij de calciumregulatie van vissen zijn dan ook andere hormonen betrokken dan bij de landdieren. *Prolactine* en *cortisol* zorgen voor een verhoogde opname van calcium door de kieuwen en remmen het verlies van calcium. Voor de hypocalcemische regulatie in vissen zorgen met name hormonen die worden gevormd en afgegeven door de *lichaampjes van Stannius*.

De lichaampjes van Stannius zijn onlangs bekend geworden doordat in de literatuur beschreven werd dat deze orgaantjes verwantschap vertonen met de bijschildklieren van de gewervelde landdieren. Antisera opgewekt tegen PTH bleken te reageren met producten uit de lichaampjes van Stannius. Verder bleken de producten uit de lichaampjes van Stannius in staat te zijn, evenals PTH, het calciumgehalte in het bloed van ratten te verhogen. Omgekeerd bleek PTH in staat te zijn het calciumgehalte in het bloed van vissen te verlagen, op dezelfde wijze als de producten van de lichaampjes van Stannius (Hoofdstuk 1). Het onderzoek beschreven in dit proefschrift heeft uitgaande van deze wetenschap geleid tot de volgende resultaten:

Homogenaten van lichaampjes van Stannius en stoffen afgegeven door deze lichaampjes hebben dezelfde invloed als PTH op het vrijmaken van calcium uit het bot van muizen. Ook de botcellen betrokken bij het vrijmaken van calcium *(osteoclasten)* worden net als door PTH, ook gestimuleerd door deze Stannius producten (Hoofdstuk 2).

Bij een verhoging van het calciumgehalte in het bloed van de forel en de paling geven de lichaampjes van Stannius een eiwit af. Dit eiwit (een *glycoprotein*) is geisoleerd uit een weefsel homogenaat van lichaampjes van Stannius waarbij gebruik is gemaakt van de suiker groepen die aan dit eiwit gebonden zijn *(glycoprotein)*. Het product is *hypocalcine* genoemd, omdat het door de Stannius lichaampjes afgegeven wordt als reactie op een verhoging van het calciumgehalte van het bloed. De *aminozuur*-samenstelling van hypocalcine van de forel is bepaald en vertoont grote overeenkomsten met de aminozuur samenstelling van hypocalcine van de zalm en de paling (Hoofdstuk 3).

Hypocalcine blijkt de stof te zijn die verantwoordelijk is voor de overeenkomst in activiteit tussen homogenaten van de Stannius lichaampjes en PTH zoals beschreven in hoofdstuk 2. De werking van hypocalcine komt het meest overeen met PTH waarvan de eerste aminozuren zijn verwijderd. Dit N-terminaal gekorte PTH maakt calcium uit het bot van muizen vrij op eenzelfde manier als hypocalcine dit doet, via de tussenkomst van Ca^{2+} als intracellulaire boodschapper (Hoofdstuk 4).

PTH is net als hypocalcine in staat het calcium gehalte in het bloed van palingen -dat tevoren door verwijdering van de lichaampjes van Stannius *(stanniectomie)* sterk verhoogd is- te verlagen. Daarbij

blijkt dat met name de ionogene calcium fractie van het bloed wordt verlaagd (Hoofdstuk 5).

Hypocalcine en PTH vertonen duidelijke overeenkomsten in activiteit. De aminozuur-samenstelling van PTH vertoont echter geen overeenkomst met de aminozuur-samenstelling van PTH. Een overeenkomst in de driedimensionale structuur van beide hormonen ligt daarom vermoedelijk ten grondslag aan de overeenkomst in activiteit.

II) Onderzoek naar het werkings mechanisme van hypocalcine.

Omdat vissen calcium opnemen via de kieuwen en omdat de lichaampjes van Stannius betrokken zijn bij de hypocalcemische regulatie werd verondersteld dat hypocalcine de opname van calcium door de kieuwen zou remmen. De opname van calcium door de kieuwen vindt plaats door speciale cellen *(chloride cellen)* in de kieuwen. Calcium komt aan de waterkant de cell binnen *(apicaal)*, gaat vervolgens door de chloride cel en gaat aan de de andere kant het bloed in *(basolateraal)*. Omdat het calciumgehalte in de cel veel lager is dan in het water loopt calcium vanuit het water mogelijk via kanaaltjes in de apicale celmembraan *(calcium channels)* de cel in. In het bloed is het calcium gehalte echter veel hoger dan in de cel. Calcium zal dus vanuit de cel het bloed ingepompt moeten worden met behulp van een calcium transporterend enzym *(Ca^{2+} transport ATPase)* (Hoofdstuk 6). Het onderzoek beschreven in het tweede deel van mijn proefschrift heeft uitgaande van deze wetenschap geleid tot de volgende resultaten:

Het weghalen van de Stannius lichaampjes uit de paling zorgt er voor dat het calciumgehalte van het bloed stijgt en gedurende enkele weken sterk verhoogd blijft. Injectie van homogenaten van Stannius lichaampjes leidt tot een daling van het verhoogde calciumgehalte in deze vissen. Met name de ionogene fractie van het bloedcalcium blijkt door de lichaampjes van Stannnius te worden verlaagd (Hoofdstuk 7).

Injectie van hypocalcine in forellen zorgt ervoor dat de opname van calcium door de kieuwen vermindert terwijl het verlies van calcium door de kieuwen onveranderd blijft. De remming van de opname van calcium door hypocalcine wordt toegeschreven aan het afsluiten van de

calcium kanalen in de apicale celmembraan van de chloride cellen (Hoofdstuk 8).

De lichaampjes van Stannius hebben twee soorten cellen, type-1 en type-2 cellen. Door bij vissen het calciumgehalte in het bloed te verhogen kon worden aangetoond dat hypocalcine mogelijk wordt afgegeven, door beide celtypen. Dit afgegeven hypocalcine zorgt waarschijnlijk voor de remming van de opname van calcium uit het water die tegelijkertijd optreedt (Hoofdstuk 9).

De resultaten uit de hoofdstukken 7, 8 en 9 hebben geresulteerd in een model voor de hormonale regulatie van de opname van calcium in zoetwater-vissen. Gesteld wordt dat zoetwater-vissen instaat zijn om uitsluitend met behulp van hypocalcine het calcium gehalte van het bloed te reguleren. Bij gewervelde landdieren zijn bij hetzelfde proces twee antagonistische functionerende hormonen betrokken: PTH en calcitonine.

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Stellingen

1) De inwaardse Ca^{2+} gradient over de apicale membraan van de Ca^{2+} transporterende cellen in de kieuwen van zoetwatervissen vormt de basis voor de hypocalcemische controle bij deze dieren.
Dit proefschrift

2) Het gebruik van de benamingen "parathyrine" en "teleocalcine" voor het hormoon uit de lichaampjes van Stannius is niet langer te verdedigen.
Contra: *Tisserand-Jochem et al. Bone and Mineral 1987 2:163*
Wagner et al. 1986 Gen Comp Endocrinol 63:481

3) De langdurige hypercalcemie na verwijdering van de hypocalcine producerende klieren in de paling duidt op de afwezigheid van functioneel vergelijkbare calciotrope hormonen.
Dit proefschrift

4) Het is onwaarschijnlijk dat de door Milet *et al. in vitro* gemeten branchiale Ca^{2+} fluxen in de paling een reële weergave vormen van de situatie *in vivo*.
Milet et al. 1980 C R Acad Sci 291:977

5) Het in de literatuur veelvuldig gebruik van de term flux ($mol/h/m^2$) in plaats van flow (mol/h) voor whole body calcium bewegingen die worden uit gedrukt in µmol/h per kg vis is onjuist.

6) De alkalische en zure fosfatasen in het serum van patienten met een prostaatcarcinoom zijn van prognostische betekenis voor de overlevingsduur van de patient en de reactie van het prostaat carcinoom op hormonale therapie.
Mulders et al. 1987 NTVG 32:1399

7) De geringe kennis van het magnesium-metabolisme in vergelijking met het calcium-metabolisme is niet het resultaat van een geringere fysiologische betekenis van magnesium maar van de problematische productie van radioactief magnesium.

8) Het feit dat mijn zoon kort na de geboorte zijn adem ingehouden zou hebben en naar de oppervlakte zou zijn komen drijven wanneer hij in het diepe gedeelte van het zwembad zou zijn 'gegooid' betekent niet dat hij biologisch gezien dicht bij de vissen zou staan.
Contra: *Little et al. Het babyboek voor vaders. Mondria, Hazerswoude*